Thesis for the Master's degree in Molecular Biosciences
Main field of study in Biochemistry

Analysis by *In Vitro* Site-Directed Mutagenesis of the PlnE Component of the Two-Peptide Bacteriocin Plantaricin EF

Bie Ekblad

60 study points

Department of Molecular Biosciences
Faculty of Mathematics and Natural Sciences
UNIVERSITY OF OSLO 09/2010

Acknowledgements

The present study, aiming at the degree of Master of Science, was performed at the Department of Molecular Biosciences, University of Oslo.

First and foremost, I want to thank Professor Jon Nissen-Meyer for accepting me in his research group, for his patience and for critical reading of this thesis. It has been a great, although sometimes frustrating journey, exploring a small piece of the bacteriocin universe!

A very special thank-you goes to my supervisor Camilla Oppegård for always answering my questions, for her patience in guiding me through obstacles along the way. I also wish to thank her for critical reading of this thesis and for all the support otherwise. It was highly appreciated. I would also like to thank the rest of the bacteriocin-group, especially Helén Haugen and Per Rogne for interesting conversations and tips during practical work, and Maren H. Backstrøm in technical staff: thank you for valuable tips during practical work and for great company during the last 6 months in the lab.

I would also like to thank my family, especially my mom for your love and encouragements and my dad for always being there if I needed to, my two beloved sisters and good friends for all your support and for believing in me.

Above all, my love and appreciations goes to my fiancé Aleksander and our daughter, Amelia. Thank you for supporting and strengthening me when I needed it the most and for reminding me that there is more to life than science! Thank you for all those moments of both joy and toil I would never have wanted to be without. I owe you a debt of gratitude I can never repay.

Abstract

Bacteriocins are ribosomally synthesized antimicrobial peptides (AMPs) and proteins produced by bacteria. The two-peptide (class-IIb) bacteriocins consist of two different peptides and optimal antimicrobial activity requires the presence of both peptides in about equimolar amounts. They exert their antimicrobial activity by rendering the target-cell membrane permeable for various small molecules. The two-peptide bacteriocin plantaricin EF consists of the two helical peptides PlnE and PlnF and is produced by some strains of lactic acid bacteria (LAB). Both PlnE and PlnF contain GxxxG motifs which might be involved in helix-helix interactions between the two peptides. Both peptides also contain aromatic tryptophan and/or tyrosine residues, which are often located in the membrane interface of membrane proteins.

In this study, variants of the PlnE peptide, which are mutated in its two GxxxG motifs (G_5xxxG_9 and $G_{20}xxxG_{24}$) and in its tyrosine residue (Y6), were constructed, since such constructs might reveal how the PlnE and PlnF peptides interact with each other and with target-cell membranes. A total of 27 mutants were constructed by *in vitro* site-directed mutagenesis and 11 of the constructed peptide variants, all involving individual residue replacements of the two glycine residues in the G_5xxxG_9 motif, were subsequently expressed, purified and assayed in complementation with the wild type PlnF peptide. The results revealed that the glycine residue at position 9 (G9) seems to be in a sterically restricted environment, since substituting G9 with large hydrophobic and large hydrophilic residues resulted in at least a 100 fold reduction in activity. It also seems that this glycine residue is in a hydrophobic environment, since replacement with a small hydrophobic residue (Ala) was less detrimental (about 10 fold reduction in activity) than replacement with a small hydrophilic residue (Ser; 60 to 70 fold reduction in activity). In contrast to G9, the glycine residue at position 5 (G5) seems not to be restricted by spatial constraints, since substituting with large hydrophobic residues (Leu/Ile) resulted in only a 2 to 6 fold reduction in antimicrobial activity. Similarly to G9, G5 also appears to be in a hydrophobic environment, since replacement with a large hydrophilic residue was detrimental (about 100 fold reduction in activity). The fact that G5 was not spatially restricted indicates that the G_5xxxG_9 motif is not a helix-helix

interacting motif. G9, being in a sterically restricted environment, may nevertheless be involved in interactions with PlnF and/or a docking protein.

Abbreviations

ABC	ATP-binding cassette
AMP	antimicrobial peptide
Da	dalton
(d) ATP	(deoxy) adenosine triphosphate
(d) CTP	(deoxy) cytidine triphosphate
(d) GTP	(deoxy) guanosine triphosphate
dH_2O	distilled H_2O
DNA	deoxyribonucleic acid
DOPG	dioleoylphosphoglycerol
DPC	dodecylphosphocholine
ΔpH	transmembrane pH gradient
dsDNA	double stranded DNA
Δψ	transmembrane electric potential
(d) TTP	(deoxy) thymidine triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
kb	kilobase
kDa	kilodalton
L	liter
LAB	lactic acid bacteria
LB	Luria-Bertani
LMGT	Laboratory of Microbial Gene Technology
L. plantarum	*Lactobacillus plantarum*
L. sakei	*Lactobacillus sakei*
mA	milli ampere
MALDI	matrix-assisted laser desorption ionization
MCS	multiple cloning site
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
MIC	minimum inhibitory concentration
µg	microgram
min	minute

µl	microliter
ml	milliliter
µM	micromolar
mM	millimolar
MRS	de Man-Rogosa-Sharpe
MS	mass spectrometry
m/z	mass/charge
NaCl	sodium chloride
NCDO	National Collection of Dairy Organisms
nm	nanomolar
OD	optical density
ON	over night
PCR	polymerase chain reaction
PCR SOEing	PCR Splicing by overlap extension
PEG	polyethylene glycol
pI	isoelectric point
PlnE	plantaricin E
PlnF	plantaricin F
RNA	ribonucleic acid
rpm	revolutions per minute
sec	second
ssDNA	single stranded DNA
TAE	tris-acetate-EDTA
TFA	trifluoroacetic acid
3D	three dimensional
TOF	time-of-flight
UV	ultra violet
V	volt
v/v	volume/volume
w/v	weight/volume

Table of Contents

1. Introduction

1.1 Antimicrobial Peptides (AMPs)

Production of antimicrobial peptides (AMPs) is an ancient and effective defence used by a wide variety of organisms to fight pathogens (*1*). AMPs have been the focus of much research during the last decades due to their potential use as therapeutic agents. Developing AMPs into new antimicrobial agents is clearly of great interest due to the increase in antibiotic-resistant bacteria resulting from large-scale use of antibiotics (*2*). There has also been great interest in developing AMPs into additives for use in animal feed and preservation of food, since the use of chemical preservatives has often undesirable side-effects (*3*).

AMPs are produced by many different kinds of organisms such as mammals (including humans), fish, birds, amphibians, insects, plants, fungi and bacteria (*4*). In animals, AMPs are effectors of innate immunity and contribute to a first line of defence during a pathogenic invasion, activated within a few hours and long before the adaptive immune system is mobilized. Hence, they prevent or delay the onset of infections (*1, 5*). Each species express its own arsenal of AMPs (*6*), which can consist of up to 35 different AMPs from various structural classes (*4*). Furthermore, experiments with knockout and transgenic mice indicate that AMPs often work in concert to combat specific infectious agents due to frequently overlapping activity spectra (*7*). Some AMPs exhibit a very selective toxicity, whereas others have a much more broad activity spectrum; the latter category includes the mammalian peptides, defensins. Some AMPs may cause harmful effects on the producer organism. As a result, many known vertebrate AMPs are secreted onto relatively inert epithelial surfaces or retained in granules of phagocytes, thereby minimizing negative effects of peptides on the host. In addition, the epithelial surfaces are normally more prone to infectious agents, placing the peptides in key positions against a potential pathogenic invasion (*8*).

As a result of the increased insights regarding the functions of AMPs, the more general term (cationic) host defence peptides (HDPs) is about to replace the term AMPs, the latter being a more narrow definition when seen in light of their emerging functions in

animals. AMPs of bacteria, often referred to as bacteriocins, may also be regarded as host defence peptides in that bacteria that produce bacteriocins achieve a competitive advantage by killing invading bacteria in their fight for nutrients (*2*). While eukaryotic AMPs are active at micromolar concentrations, bacteriocins are active at nanomolar concentrations (*2*). Generally, the latter also have a more narrow target-cell spectrum than AMPs of eukaryotes (*2*).

AMPs are gene-encoded, ribosomally synthesized peptides (*2*). As a result, 'peptide' antibiotics are not considered to be AMPs because their synthesis is mediated by a unique system composed of multifunctional enzymes rather than via ribosomes (*2, 9*). Although antimicrobial peptides make up a diverse group as judged by their primary structure, some features seem to be in common; they are often membrane permeabilizing, cationic (with a net positive charge ranging from +2 to +9) and amphiphilic or hydrophobic when folded (*9, 10*). These properties are of importance when it comes to initial contact with a pathogen since they enable electrostatic and hydrophobic interactions between the peptide and the bacterial membrane, which consists of negatively charged and hydrophilic head groups as well as a hydrophobic core (*4, 6*).

1.2 Bacteriocins

Bacteriocins represent a diverse and abundant group of antimicrobial peptides and proteins produced by bacteria. They were first discovered in *Escherichia coli* (*E. coli*) as substances, named colicins, that inhibited the growth of some strains of *E. coli* (*11*). After the discovery of colicins, a great number of bacteriocins have been identified in many bacteria from different taxonomic branches and habitats (*12, 13*). Gram-negative bacteria often produce protein bacteriocins larger than 20 kDa whereas Gram-positive bacteria, including lactic acid bacteria, most often produce peptide bacteriocins smaller than 6 kDa (*2*).

Lactic acid bacteria (LAB) and their bacteriocins have been the focus of much research due to their beneficial influence in fermented foods and the fact that they are generally regarded as safe (*10, 14*). Some LAB are also part of the natural microflora in humans

and are thought to be beneficial to human health, reflecting the increased marketing of LAB-containing probiotic products (*15*).

The bacteriocin nisin has been formally used as a food preservative for over 30 years and is currently the only bacteriocin licensed as a food additive (in a wide variety of products) in over 48 countries (*16*). Pediocin PA-1 has also been commercially exploited, particularly in meat products, and as a pediocin-containing powder in dairy products (*17*). Although bacteriocin-producing LAB have been deliberately used in food for only a few decades, humans have probably benefited from their influence in fermented food for thousands of years (*18*). LAB bacteriocins have also given promising results in medical applications. A recent study showed that oral intake of *Lactobacillus salivarius* UCC118, producing the bacteriocin ABP118, conferred a protective effect in mice against the food-borne pathogen *Listeria monocytogenes* (*19*). Furthermore, a class-IIa bacteriocin (section 1.3), enterocin CRL35, has been shown to behave as an antiviral agent against herpes simplex viruses (*20*).

1.3 Classification of LAB Bacteriocins

The LAB bacteriocins can be divided into two main classes, class-I and class-II (Fig 1.1). Class-I consists of the lantibiotics, which are lanthionine-containing post-translationally modified bacteriocins. Class-II consists of the non-lanthionine-containing bacteriocins (*10, 18*). The lantibiotics (i.e. class-I bacteriocins) contain the thioether-linked amino acids lanthionine and 3-methyllanthionine. Up to 15 other modified residues (such as dehydroalanine, dehydrobutyrine and D-alanine) have also been identified in some lantibiotics (*10, 21*). Lanthionine and 3-methyllanthionine are formed by a two-step mechanism which involves dehydration of serine (to lanthionine) or threonine (to 3-methyllanthionine) in the first step followed by a thioether-linkage to a sulphydryl group of a cysteine residue in the second step, thus creating the intramolecular rings common to all lantibiotics (*21*). Both one- and two-component (two-peptide) lantibiotics have been identified (*22*). Examples of class-I bacteriocins (lantibiotics) are nisin, subtilin and lactocin S (*21*).

Class-II, the non-lanthionine-containing bacteriocins, which are small and heat-stabile, may be further subdivided into four subclasses, class-IIa, class-IIb, class-IIc and class-IId (*18*). The class-IIa bacteriocins are often referred to as the pediocin-like bacteriocins because the first identified bacteriocin in this subclass was pediocin PA-1 (*23*). These peptide bacteriocins are active against pathogenic bacteria such as *Staphylococcus aureus* and *Listeria monocytogenes*. They contain between 37 to 48 amino acid residues and have the consensus sequence YGNGV(X)C(X)$_4$C(X)V(X)$_4$A (where X is any amino acid) in the N-terminal region (*23*). Class-IIc consists of the cyclic bacteriocins in which the C- and N-terminal ends are covalently linked. Class-IId contains a heterogeneous group of bacteriocins as the class consists of all one-peptide, non-cyclic bacteriocins whose sequences have no similarity to the pediocin-like bacteriocins (*10*, *18*). Class-IIb consists of the two-peptide bacteriocins whose optimal antimicrobial activity requires the presence of two different peptides in about equimolar amounts (*24*). The two-peptide bacteriocin plantaricin EF belongs to this subclass and is also the focus of this thesis. As such, this subclass will be discussed more thoroughly in the next sections.

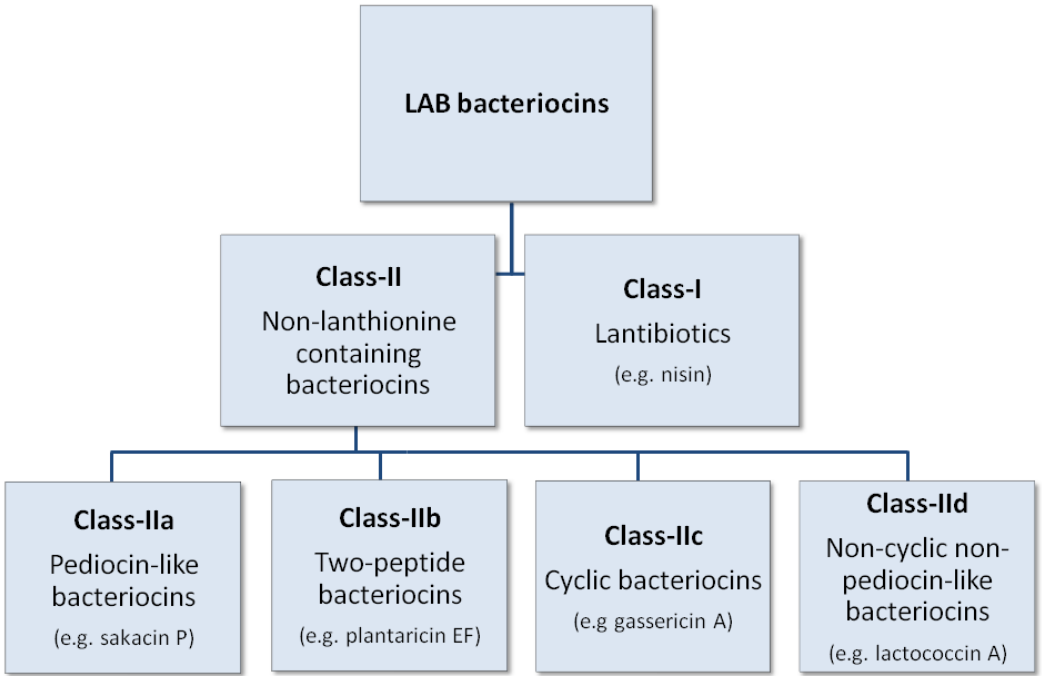


Fig 1.1 Classification of the LAB bacteriocins. The bacteriocins are divided into two main classes; class-I (the lantibiotics) and class-II (the non-lanthionine containing bacteriocins). The latter can be further divided into four subclasses. Class-IIa consists of the pediocin-like bacteriocins. The two-peptide bacteriocins are categorized in class-IIb. Class-IIc consists of the cyclic bacteriocins, while the non-cyclic non-pediocin-like bacteriocins are placed in class-IId.

1.4 Class-IIb Bacteriocins

Both peptides of two-peptide (class-IIb) bacteriocins are usually cationic and contain hydrophobic and/or amphiphilic regions (*24*, *25*). The net positive charge is important for their preferential binding to the negatively charged surface of bacteria, such as lipopolysaccharides in Gram-negative bacteria and wall-associated teichoic acids in Gram-positive bacteria (*9*). Since their discovery in 1992 (*26*), more than 15 two-peptide bacteriocins have been identified and genetically characterized (Table 1.1). The specific antimicrobial activity is generally highest when both peptides are present in about equal amounts. Individually, the peptides of two-peptide bacteriocins exert only low, if any, antimicrobial activity. For instance, when present together, the two complementary peptides (LcnG-α and LcnG-β) of the two-peptide bacteriocin lactococcin G are active at less than nanomolar concentrations, but they show no activity individually at micromolar concentrations (*27*).

Table 1.1 An overview of characterized class-IIb bacteriocins:

Bacteriocin[a)]	Producer strain	References
Lactococcin G [b)]	*Lactococcus lactis* LMGT 2081	(*26*)
Lactococcin Q [b)]	*Lactococcus lactis* QU 4	(*28*)
Enterocin 1071 [b)]	*Enterococcus faecalis* FAIR-E 309	(*29*)
	Enterococcus faecalis BFE 1071	(*30*, *31*)
Enterocin C[c)]	*Enterococcus faecalis* C901	(*32*)
Enterocin X	*Enterococcus faecium* KU-B5	(*33*)
Plantaricin EF [d)]	*Lactobacillus plantarum* C11	(*34*, *35*)
Plantaricin JK [d)]	*Lactobacillus plantarum* C11	(*34*, *35*)
Plantaricin S	*Lactobacillus plantarum* LPCO10	(*36*, *37*)
Plantaricin NC8	*Lactobacillus plantarum* NC8	(*38*)
Lactocin 705	*Lactobacillus casei* CRL 705	(*39*)
Lactacin F	*Lactobacillus johnsonii* VPI11088	(*40*, *41*)
Brochocin C	*Brochothrix campestris* ATCC 43754	(*42*)
Thermophilin 13	*Streptococcus thermophilus* Sfi13	(*43*)
ABP-118	*Lactobacillus salivarius* subsp. *salivarius*	(*44*)
Salivaricin P	*Lactobacillus salivarius* DPC6005	(*45*)
Mutacin IV	*Streptococcus mutans* UA140	(*46*)

[a)] Lactococcin MMT24 (*47*), lactococcin MN (*48*) and leucocin H (*49*) are not included in the table. Lactococcin MMT24 has not been sequenced whereas lactococcin MN has only been sequenced in the preform. Leucocin H has only been partially sequenced.

[b)] Lactococcin G, lactococcin Q and enterocin 1071 share high sequence similarities enabling high activity when combining one peptide from one of these bacteriocins with the complementary peptide from another of these bacteriocins (*24*).

[c)] Enterocin C differs only in one amino acid compared to enterocin 1071, but has a higher inhibitory activity as well as a much broader activity spectrum (*32*).

[d)] Plantaricins EF and JK are produced by several different *Lactobacillus plantarum* strains in addition to the strain C11 (*25, 50*).

1.4.1 Expression and Secretion of Two-Peptide Bacteriocins

For all genetically characterized two-peptide bacteriocins, at least five genes are required for bacteriocin production: the two genes encoding the two peptides constituting the bacteriocin, a gene encoding the immunity protein that protects the bacteriocin-producing bacteria from its own bacteriocin, a gene encoding a dedicated ATP-binding cassette (ABC) transporter that exports the bacteriocin out of the cell, and a gene encoding an accessory protein whose exact function is unknown, but it may be involved in immunity toward and/or secretion of the bacteriocin (*27*). These genes are localized in either the same or in two nearby operons (*2, 27*). The two genes encoding the two peptides constituting the bacteriocin are always located next to each other on the same operon along with the gene encoding the immunity protein (*24*). The genes encoding the dedicated ABC transporter and the accessory protein are located either in the same operon as the bacteriocin genes (as is the case for lactococcin G (*27*)) or in a nearby operon (as is the case for enterocin 1071 (*31*) and plantaricins EF and JK (*35*)) (*51*) . In addition to the results showing that both bacteriocin peptides need to be present to attain a potent antimicrobial effect (*24*), this organization of genes encoding the two bacteriocin peptides in the same transcriptional unit (meaning that the two peptides are likely to be produced in about equal amounts) and the fact that there is only one immunity protein for a specific two-peptide bacteriocin, strongly suggest that the two bacteriocin peptides function as one antimicrobial entity (*10, 24*). This is in contrast to two one-peptide bacteriocins acting synergistically at two different sites on the target cell. In fact, a recent study has shown that the two complementary peptides of lactococcin G (LcnG-α and LcnG-β) are recognized as one physical entity by their cognate immunity protein (*52*).

The two peptides of all the two-peptide bacteriocins that have been characterized are synthesized with a double-glycine type leader sequence at the N-terminal end, the length of the leader being 15 to 30 amino acid residues (*24*). The N-terminal double glycine leader is cleaved off at the C-terminal side of the two glycine residues by the ABC transporter concomitantly with export of the peptides. This ABC transporter has an N-terminal extension of about 150 amino acid residues which has been shown to possess proteolytic activity (*51*). The N-terminal glycine leader seems to function both

as a recognition signal for the ABC transporter and possibly also to keep the bacteriocin in an inactive state until it has been secreted (*53*).

1.4.2 Regulation of Two-Peptide Bacteriocin Expression

Some two-peptide bacteriocins (such as lactococcin G (Nissen-Meyer, J., personal communication)) seem to be produced constitutively, whereas others (such as ABP-118 (*44*) and plantaricins EF and JK (*35, 54*)), are regulated by a so-called three-component regulatory system. The three-component regulatory system consists of a peptide pheromone (induction factor), a histidine protein kinase and a response regulator (*54-56*). See Fig 1.2 for a schematic overview of regulation of two-peptide bacteriocins. The peptide pheromone is produced with an N-terminal leader sequence of the double-glycine type which is cleaved off upon export across the membrane by the same ABC transporter that processes and secretes the bacteriocin. The genes encoding the peptide pheromone, the histidine protein kinase and the response regulator are located in the same operon (*54-56*). The expression of the genes is presumably triggered in a cell-density dependent manner. The peptide pheromone has a low, but constitutive secretion during growth. When reaching a threshold concentration, the peptide pheromone interacts with the membrane-bound histidine protein kinase that result in autophosphorylation of the latter and subsequent phosphorylation of the cytoplasmic response regulator. The phosphorylated response regulator binds in turn to DNA and activates the operons necessary for transcription of the genes involved in production and secretion of the bacteriocin (*56, 57*). The activation of the histidine protein kinase also seems to increase the production of the peptide pheromone itself, thereby creating an autoinduction loop ensuring a rapid and massive burst of bacteriocin production (*56*).

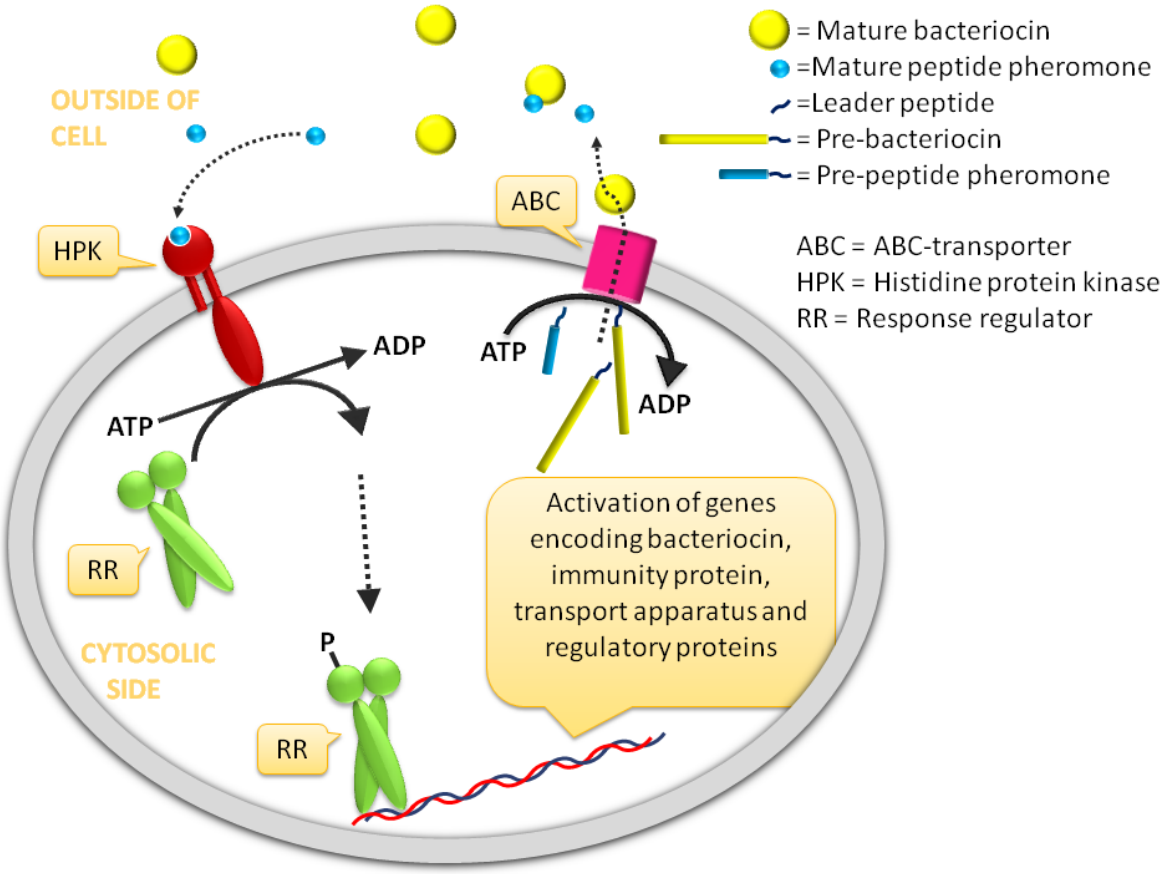


Fig 1.2 A simplified, schematic representation of the regulation of bacteriocin biosynthesis. The production of some two-peptide bacteriocins are regulated through a three-component regulatory system including a peptide pheromone, a histidine protein kinase and a response regulator. The leader peptides of both the peptide pheromone and the bacteriocin are cleaved off concomitant with transport to the extracellular medium by a dedicated ABC-transporter. Once outside the cell, the peptide pheromone triggers an autophosphorylation of the histidine protein kinase which in turn phosphorylates the response regulator. The response regulator then binds to the DNA and activates transcription of the genes necessary for production and secretion of the bacteriocin. The bacteriocin and the peptide pheromone are not structured in aqueous solutions (*58, 59*) although this figure represents it that way.

1.4.3 Structure and Mode of Action

As already mentioned, two-peptide bacteriocins are usually cationic and contain hydrophobic and/or amphiphilic regions. Structural studies performed by circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy have been carried out on three two-peptide bacteriocins, lactococcin G, plantaricin EF and plantaricin JK (*58, 60-63*). The CD studies showed that all the peptides had a non-structured conformation in aqueous solutions, but upon exposure to membrane-mimicking environments, such as micelles and negatively charged liposomes, they adopted mainly an α-helical structure. Furthermore, additional α-helical structuring was obtained in both

complementary peptides when they were premixed before they were added to liposomes, which might imply that the two peptides interact with each other upon contact with target membranes (*58, 60*). Additional structuring was not observed when the two peptides were added to liposomes one after the other (*58, 60*). It was also shown for lactococcin G that no activity is obtained when cells are first treated with one of the two lactococcin G peptides and then mixed with cells treated with the other lactococcin G peptide (*64*). Antimicrobial activity is, however, obtained when cells are treated with one of the peptides and then extensively washed before adding the complementary peptide (*64*). These results may suggest that the individual peptides first bind to the target-cell surface and then interact with the complementary peptide before being fully embedded in the membrane (*64*). It also seems that once a peptide is bound to the target membrane it is unable to diffuse to another membrane (*10, 64*).

NMR studies have revealed the 3D structures of the peptides that constitute lactococcin G (*62*), plantaricin EF (*61*) and plantaricin JK (*63*). All 6 peptides formed mainly amphiphilic α-helices with more flexible regions in the N- and C-terminal halves when exposed to membrane-mimicking environments. Although the amphiphilic α-helix seems to be an important structural motif in class-IIb bacteriocins, it appears that some bacteriocins in this subclass may adopt a β-sheet structure as may be the case for brochocin C (*24*). 3D structural studies when both complementary peptides are present have not yet been performed.

After insertion into the membrane, all two-peptides whose mode of action has been studied (this includes plantaricins EF and JK (*65*), lactococcin G (*64, 66*), lactacin F (*67*), lactocin 705 (*68*) and thermophilin 13 (*43*)) render the membranes of target cells permeable to small molecules, eventually leading to cell death. The specificity as to what ions they conduct across the membrane of target cells varies among the bacteriocins. For instance, lactacin F permeabilizes membranes for K^+ and inorganic phosphate (*67*), lactococcin G permeabilizes the membrane for different monovalent cations such as K^+, Na^+, Cs^+ and Rb^+ (*64*), but does not conduct H^+ as lactococcin G has no effect on the transmembrane pH gradient (ΔpH) (*64, 66*). Plantaricin EF seems to conduct monovalent cations more effectively than plantaricin JK (such as Rb^+ and

choline as well as H^+), whereas plantaricin JK seems to conduct anions more effectively than plantaricin EF (*65*).

The high potency of two-peptide bacteriocins (antimicrobial activity is observed at picomolar to nanomolar concentrations (*63, 69*)) and their ability to differentiate between various molecules they conduct across the membrane, suggest that these bacteriocins create specific pores rather than an unspecific disruption of membranes (*24*). Questions about possible interactions between bacteriocins and receptors in the target-cell membrane have been raised. The lantibiotic, nisin, has been shown to specifically interact with lipid II, a central component in bacterial cell wall synthesis, upon binding to the bacterial membrane (*70*). Moreover, a recent study showed that lactococcin A, a class-IId bacteriocin, and pediocin-like bacteriocins (class-IIa) bind to part of the mannose phosphotransferase system (man-PTS), a system which is important for the uptake of mannose in bacteria (*71*). The study also showed that the cognate immunity protein to lactococcin A binds to the bacteriocin-receptor complex in lactococcin A-producing bacteria and thereby prevents the bacteria from being killed by lactococcin A. No such interaction was detected between bacteriocins, the man-PTS and the cognate immunity proteins of the two-peptide bacteriocins plantaricin EF and lactococcin G (*71*).

A recent study on lactococcin G and its cognate immunity protein (*52*) revealed that the lactococcin G immunity protein confers resistance toward the highly similar bacteriocin enterocin 1071 in an *Enterococcus* strain, but not in a *Lactococcus* strain to the same extent, indicating that also the lactococcin G immunity protein depends on a cellular component (possibly the bacteriocin receptor) in order to function (*52*). It has indeed been suggested that lactococcin G (as well as other two-peptide bacteriocins) function in an analogous manner as lactococcin A and the pediocin-like bacteriocins; i.e. by binding of lactococcin G to an integrated membrane protein whereby the lactococcin G immunity protein recognizes and binds to the bacteriocin-receptor complex, making the lactococcin G-producing bacteria immune toward its own bacteriocin (*72*).

1.5 The Two-Peptide Bacteriocin Plantaricin EF

Various bacteriocin-producing *Lactobacillus plantarum* (*L. plantarum*) strains have been isolated from vegetable and fermented sources as well as from human mucosa, and some of these strains produce the bacteriocin plantaricin EF (*25*). Plantaricin EF consists of the two peptides plantaricin E (PlnE) and plantaricin F (PlnF) and was identified by DNA and protein similarity searches in 1996 along with another two-peptide bacteriocin, plantaricin JK, both produced by *L. plantarum* C11 (*35*). The fact that they possessed antimicrobial activity was verified two years later in activity assays (*34*). Although the PlnF peptide showed some activity when tested individually (at a concentration of 5 µM) their activity is increased at least 10^3 times when combined and optimal activity requires both PlnE and PlnF peptides in about equal amounts. The PlnE peptide did not exert any antimicrobial activity at concentrations up to 13 µM when tested without the PlnF peptide (*34, 58*). Both plantaricin EF and plantaricin JK bacteriocins display quite narrow inhibition spectra and are mainly active against closely related species of *L. plantarum (25).*

1.5.1 Induction of Plantaricin EF Expression

The production of the two-peptide bacteriocins plantaricins EF and JK and their cognate immunity proteins is controlled by a three-component regulatory system as described in section 1.4.2. The *pln* loci responsible for bacteriocin production and regulation in *L. plantarum* C11 are organized into five inducible operons (Fig 1.3) where the inducer is the peptide pheromone plantaricin A (PlnA) (*73*). PlnA was previously thought to be a bacteriocin because of its antimicrobial activity and the fact that it contained a double-glycine type leader sequence (*74-76*), but was later found to possess both bactericidal and pheromone activities with its main biological function being its pheromone activity (*59*). The results showed that the all-D-enantiomer of a truncated version of PlnA remained bactericidal *in vivo*, but the pheromone activity was lost, indicating that its pheromone activity involves chiral interactions with a receptor protein (presumably the histidine protein kinase), whereas its antimicrobial activity involves a non-chiral interaction with membrane lipids (*77*).

1.5.2 Regulation and Expression of Plantaricin EF

As illustrated in Fig 1.3, five operons are important for regulation and expression of plantaricins EF and JK and the transcription of these operons are all induced by the peptide pheromone PlnA. Two of the five PlnA-induced operons, *plnEFI* and *plnJKLR*, encode the two two-peptide bacteriocins plantaricin EF (and its cognate immunity protein PlnI) and plantaricin JK (and its cognate immunity protein PlnL), respectively (*35*). The putative protein encoded by the *plnR-g*ene has a yet unknown function, but it might be required for the protein PlnL to confer immunity against plantaricin JK (*78*). The two genes *plnGH* that are located in the transport operon, *plnGHSTUVW*, encode an ABC transporter (PlnG) and an accessory protein (PlnH) involved in the maturation and export of peptides containing double-glycine type leader sequences (*25, 35*). What role in bacteriocin biosynthesis the remaining genes in this operon (*plnSTUVW)* may have is still unknown, but they show significant sequence similarity to each other as well as to *plnI, plnL* and *plnP* (the latter from the operon with unknown function) and to the Abi protein family, a family of proteins with putative proteolytic activity (*25, 78*). The regulatory operon, *plnABCD*, codes for the peptide pheromone PlnA, a histidine protein kinase (PlnB) and two response regulators, PlnC and PlnD. The fact that this operon contains two response regulators is in contrast to what is observed with many other known three-component regulatory systems, including those of other *L. plantarum* strains where only one response regulator has been identified (*25, 79*). The functions of the regulators PlnC and PlnD are complex and their action is still not fully understood, but they seem to play a part in the regulation of bacteriocin biosynthesis by an interplay between transcriptional activation (mediated by PlnC) and repression (mediated by PlnD) of the genes involved in bacteriocin production and regulation. Both regulators bind to conserved tandem repeats located in the promoters of all five operons (*80*). The functions of the genes located in the final PlnA-induced operon, *plnMNOP*, remain to be determined. The PlnN peptide was originally thought to be a bacteriocin because the *plnN*-gene encoded a double-glycine type leader sequence and the mature peptide had a putative amphiphilic α-helix, but synthetic PlnN exhibited no bactericidal activity when assayed against different indicator strains (*34*).

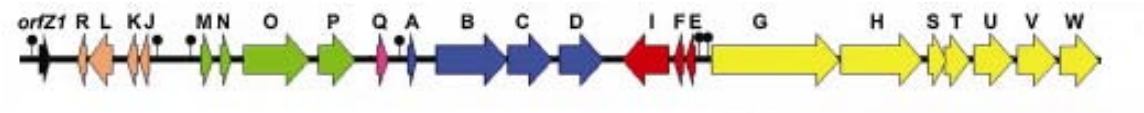


Fig 1.3 Genetic map of the *pln* locus containing 5 PlnA-induced operons required for production and regulation of the two-peptide bacteriocins plantaricin EF and plantaricin JK. The entire *pln* locus is approximately 18 kb long (NCBI accession X94434.2) and is organized into 5 inducible operons consisting of a total of 22 ORFs (*25*). The operons *plnEFI* and *plnJKLR* encode the two-peptide bacteriocins plantaricin EF and plantaricin JK as well as their cognate immunity proteins PlnI and PlnL, respectively. *PlnABCD* encodes the peptide pheromone PlnA, the histidine protein kinase PlnB and two response regulators, PlnC and PlnD, whereas the genes encoding the ABC transporter (PlnG) and the accessory protein (PlnH) are located in the operon *plnGHSTUVW*. The rest of this operon along with the *plnMNOP*-operon encode putative proteins with unknown functions as is also the case for the genes *orfZ1* (black arrow) and *plnQ* (pink arrow) *(25)*. Lollipops indicate regulated promoters. The figure is adapted from Diep *et al.* (*25*).

The two peptides of the plantaricin EF bacteriocin (as well as plantaricin JK) are synthesized as prepeptides containing the typical class-II AMP double-glycine type leader sequence in their N-terminal end (Fig 1.4). Concomitant with transport, this leader sequence is cleaved off at the C-terminal end of two glycine residues by the gene-product of *plnG* encoding an ABC transporter (*35*). The length of the amino acid sequences of the leader peptides of PlnE and PlnF are 23 and 18 residues long, respectively. The mature peptide of PlnE contains 33 amino acid residues with a theoretical molecular weight of 3545 and an isoelectric point (pI) of 11.57. The mature peptide of PlnF contains 34 amino acid residues with a molecular weight of 3703 and a pI of 10.27 (molecular weights and pIs have been computed based on their mature amino acid sequences from the web-site: http://www.expasy.org/tools/, 'Compute pI/Mw'; august 2010) . The sequences are adapted from EMBL Nucleotide Sequence Database, accession number X94434.2.

PlnE: MLQFEKLQYSRLPQKKLAKISGG▼FNRGGYNFGKSVRHVVDAIGSVAGIRGILKSIR

PlnF: MKKFLVLRDRELNAISGG▲VFHAYSARGVRNNYKSAVGPADWVISAVRGFIHG

Fig 1.4 Amino acid sequences of the preforms of PlnE and PlnF from N- to C-terminal end. The two glycine residues of the double-glycine type leader sequence are marked in green and the cleavage site (cleaved by the ABC transporter) is marked with a black triangle. The C-terminal side of this triangle indicates the mature peptide. The glycine residues in the three GxxxG motifs (two in PlnE and one in PlnF) are marked in red.

1.5.3 Structure and Mode of Action of Plantaricin EF

When in contact with the target-cell membrane, the plantaricin EF bacteriocin induces pore-formation, causing an efflux of monovalent cations such as choline, Rb^+ and H^+, which eventually leads to dissipation of the transmembrane electric potential ($\Delta\psi$) and subsequent cell death (*65*). Plantaricin EF also dissipates the ΔpH, but to a much lesser extent than dissipation of the $\Delta\psi$. It should be mentioned that the plantaricin JK bacteriocin dissipates the ΔpH much more effectively than the $\Delta\psi$ (the opposite of what is observed with plantaricin EF) by membrane leakage of anions (*65*). It also seems that plantaricin JK more effectively inhibits growth of susceptible cells than does plantaricin EF. This might imply that ΔpH is more important for cell viability, leading to a drop in the intracellular pH and thereby inhibition of metabolism and depletion of the cellular ATP pool, resulting in a more rapid cell death (*65*).

As mentioned in section 1.4.3, CD analyses of PlnE and PlnF peptides showed that both adopt an amphiphilic α-helix upon contact with membrane-mimicking environments such as dodecylphosphocholine (DPC) micelles and negatively charged dioleoylphosphoglycerol (DOPG) liposomes, but that they are unstructured in aqueous solutions (*58*). Additional structuring was induced by interpeptide interactions, but only when they were premixed and then exposed to negatively charged liposomes. No interpeptide-induced structuring was seen when they were simultaneously exposed to trifluoroethanol or DPC micelles, the latter possibly due to the fact that micelles are much smaller than liposomes and therefore also possess a steeper curvature (*61*). The above results indicate that (i) preferential binding to target membranes is due to electrostatic interactions between the cationic peptide and the negatively charged membrane surface and that (ii) PlnE and PlnF induce additional structuring in each other upon contact with the target membrane. The 3D structures of the individual PlnE and PlnF peptides have been determined by NMR spectroscopy in the presence of DPC micelles (*61*). Figs 1.5 A and 1.5 B show the structures of both PlnE and PlnF, respectively, along with Edmundson α-helical wheel presentations of parts of their α-helical region (*58*). Earlier CD analyses and Edmundson wheel predictions of amphiphilic helices within the two peptides of PlnE and PlnF were found to include residues 1 to 24 and 15 to 34, respectively (*58*). This prediction turned out to be in good

agreement with the results obtained by NMR spectroscopy. PlnE was shown to form two separate amphiphilic α-helices, one α-helix spanning from residue 10 to 21 and the other from residue 25 to 31, while PlnF forms one long α-helix from residue 7 to 34 with a proline residue in the middle resulting in a kink of 38 ± 7 degrees at the position of the proline. The helix in PlnF was hydrophobic in the C-terminal part whereas the N-terminal part (between residue 11 and 16) was very polar and because of this, the peptide is not likely to transverse a membrane without interacting with other molecules, indicating a possible interaction with an unknown membrane protein (*61*).

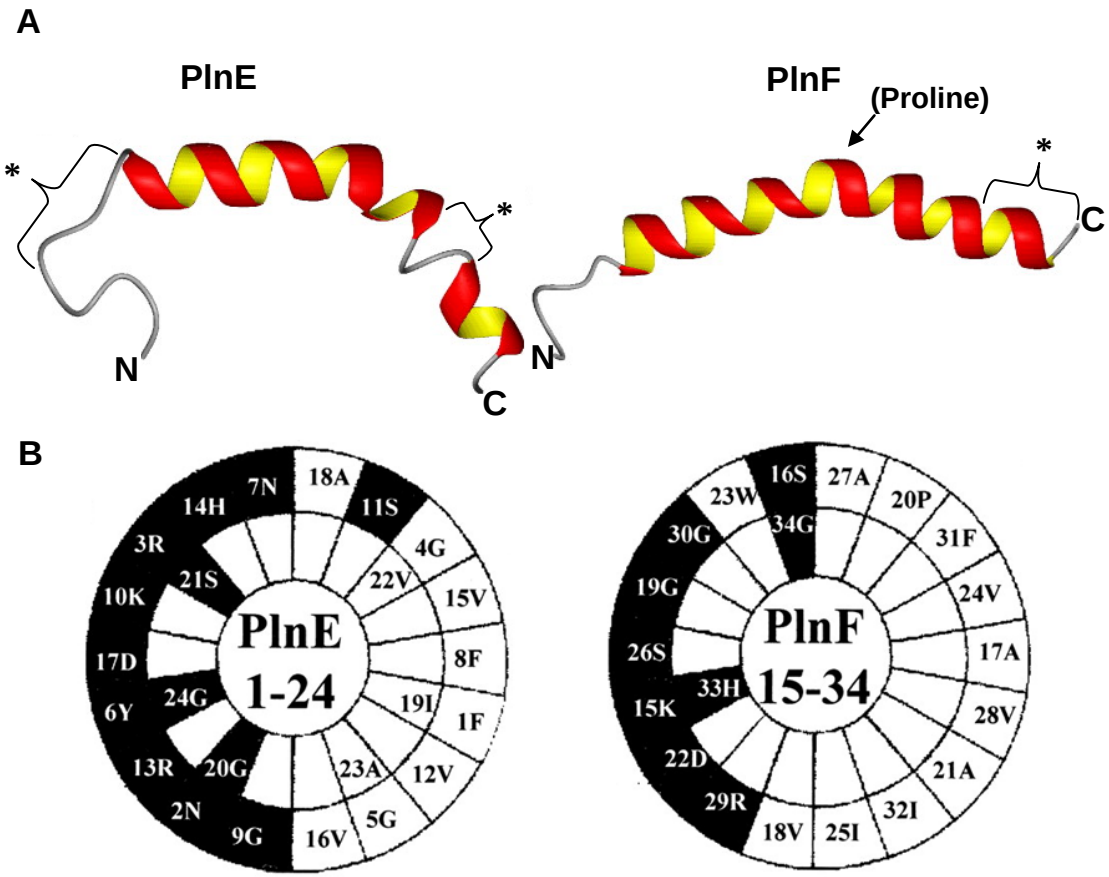


Fig 1.5 A) The NMR structures of the individual plantaricin EF peptides and B) Edmundson α-helical wheel presentations of parts of their α-helical region. A) The NMR structure of PlnE and PlnF was determined in the presence of DPC micelles. The PlnE peptide forms two α-helices from residue 10 to 21 and from 25 to 31. The PlnF peptide forms one long α-helix spanning from residue 7 to 34. The three asterisks indicate the GxxxG motifs and they are located in the flexible regions of the two peptides (see below, section 1.5.4). The figure is adapted from Fimland *et al. (61)*. **B)** The Edmundson α-helical wheel presentations of the peptides PlnE and PlnF show that both peptides can adopt an amphiphilic α-helical structure. The polar residues are coloured in black whereas the non-polar residues are coloured in white. Glycine residues are treated as being neutral with respect to polarity and are therefore coloured in both black and white depending on the position in the wheel. The figure is adapted from Hauge *et al. (58)*.

1.5.4 The GxxxG Motif

One or more GxxxG motifs (where x is any amino acid) have been identified in all two-peptide bacteriocins discovered so far (*27*). The presence of GxxxG or 'GxxxG-like' (such as AxxxA and SxxxS) motifs has emerged as a characteristic signature of helix-helix interactions in many transmembrane proteins (*81*). When these motifs are positioned in an α-helical segment, the small glycine residues are on the same side of the helix and thus form an almost flat contact surface, enabling close interhelical contact. This will allow for interpeptide van der Waals interactions as well as formation of stabilizing interhelical backbone Cα−H···O hydrogen bonds (*82, 83*). The GxxxG motifs are thought to be important for the interhelical interactions between two-peptide bacteriocins (*27*), although the motifs by themselves are probably not sufficient for creation of the helix-helix interactions (*84*). Based on site-directed mutagenesis studies and NMR analysis, a structural model of the two-peptide bacteriocin lactococcin G (which consists of the two peptides LcnG-α and LcnG-β) when integrated in target-cell membranes has been proposed (Fig 1.6) (*10, 62, 72*). Site-directed mutagenesis analysis indicates that two of the three putative helix-helix interacting GxxxG motifs in lactococcin G are important in helix-helix interactions (*72*). Replacement of the glycine residues in these motifs with larger residues (both hydrophobic and hydrophilic) reduced the antimicrobial activity dramatically, whereas replacement with small amino acids such as alanine (hydrophobic) and serine (hydrophilic) were reasonably well tolerated (*72*).

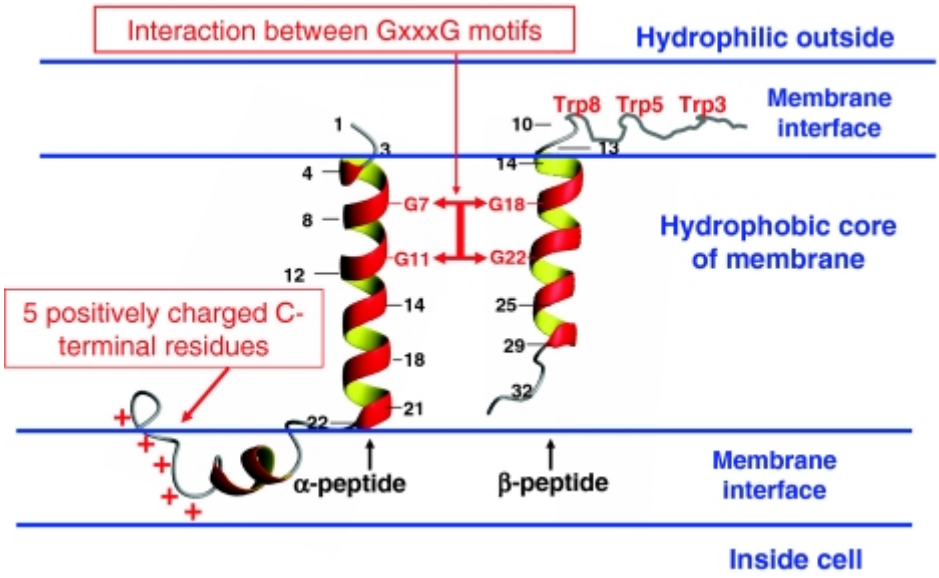


Fig 1.6 The proposed structural model of lactococcin G and its orientation in target-cell membranes (an example of how the two peptides of two-peptide bacteriocins may interact with each other and the target membrane). The two peptides seem to interact through the G_7xxxG_{11} motif in LcnG-α and the $G_{18}xxxG_{22}$ motif in LcnG-β, forming a parallel transmembrane helix-helix structure. Further stabilization of the structure and orientation/anchoring in the membrane may be favoured by the positively charged C-terminal end of LcnG-α and the tryptophan-rich N-terminal end of LcnG-β, positioned at each side of the membrane interface, as well as the C-terminal Trp residue (W32) in LcnG-β (*72*). The figure is adapted from Nissen-Meyer *et al.* (*27*).

The PlnE peptide contains two putative helix-helix interacting GxxxG motifs, one positioned from residue 5 to 9 (G_5xxxG_9) and one from residue 20 to 24 ($G_{20}xxxG_{24}$), whereas the PlnF peptide contains one such motif from residue 30 to 34 ($G_{30}xxxG_{34}$). All three GxxxG motifs are marked with an asterisk in Fig 1.5 A. Based on the results from NMR spectroscopy, these regions are flexible, but will become more rigid upon helix-helix interactions between the two peptides. This may possibly explain the increased structuring which has been observed by CD analysis when PlnE and PlnF are mixed before exposure to liposomes (*58, 61*). Two plausible interaction models, describing a parallel and an anti-parallel orientation of the two peptides, have been proposed (*85*). The first possibility is that the two peptides interact in a parallel fashion leading to a pairing between the $G_{20}xxxG_{24}$ motif in PlnE and the $G_{30}xxxG_{34}$ motif in PlnF, which in turn will enable charge-charge interactions between different amino acid residues in both peptides (*85*). This will also allow the G_5xxxG_9 motif in PlnE to come in close contact with the flat surface of PlnF at the region between residues 16 and 20 where the proline residue (P20) is positioned (Fig 1.5 A). There are also two small

amino acid residues located in this area of PlnF, serine (S16) and glycine (G19) (*85*). A second possibility is that the two peptides interact in an anti-parallel manner upon interaction between the G_5xxxG_9 motif in PlnE and the flat surface of PlnF, which also will enable interpeptide charge-charge interactions (*85*). See Fig 5.1, section 5.2 for an illustration of the two putative helix-helix interacting models.

1.5.5 Tryptophan and Tyrosine Residues

Compared to the hydrophobic core of the membrane, the membrane interface comprises a complex environment offering a number of different possibilities of non-covalent interactions to which the residue side chains of membrane proteins and peptides can interact (*86*). The aromatic residues tryptophan and tyrosine are known to be preferentially located in the membrane interface of membrane proteins, suggesting an important role in promoting binding and folding of these (*86-89*). Indeed, results from site-directed mutagenesis studies regarding lactococcin G (*72*) (see Fig 1.6 for a cartoon model of lactococcin G embedded in a target-cell membrane) showed that substituting the tryptophan residue at position 32 in the LcnG-β peptide with both large hydrophobic and large hydrophilic residues were relatively detrimental, whereas substituting with an aromatic residue (such as tyrosine and phenylalanine) was almost as good as wild type activity (*72*). This preference for aromatic residues, but not for hydrophobic residues, indicate that this tryptophan residue is positioned in the membrane interface (*72*).

The mature PlnE peptide contains one tyrosine residue at position 6 (Y6; Fig 1.4) and the mature PlnF peptide contains two tyrosine residues (Y5 and Y14) and one tryptophan residue (W23). The role these residues may play in correct positioning of plantaricin EF in target-cell membranes remains to be elucidated.

2. Aim of Study

An overall aim of structure-function characterization of the two-peptide bacteriocin plantaricin EF is to determine how the two bacteriocin peptides, PlnE and PlnF, interact with each other and how they interact with target membranes.

Most, if not all, two-peptide bacteriocins discovered so far contain a putative amphiphilic or hydrophobic α-helix as well as one or more GxxxG motifs. Such motifs are known to mediate helix-helix interactions in membrane proteins and one might postulate that these motifs are important for the alleged interhelical packing between the two peptides of two-peptide bacteriocins upon interaction with target-cell membranes. Tryptophan and tyrosine residues are often found in membrane proteins and are preferentially located in the membrane interface. Mutational studies of the GxxxG motifs and aromatic tyrosine and tryptophan residues in two-peptide bacteriocins may thus provide insight into the structure of two-peptide bacteriocins and the orientation of the structure in target-cell membranes. The PlnE peptide contains two GxxxG motifs (G_5xxxG_9 and $G_{20}xxxG_{24}$) and one tyrosine (Y6) residue, but no tryptophan residues.

The aim of this study was to construct variants of the PlnE peptide that are mutated in the two GxxxG motifs and in the tyrosine residue, since such constructs might reveal how the PlnE and PlnF peptides interact with each other and with target-cell membranes. The glycine residues in both GxxxG motifs in PlnE were thus to be individually replaced with a small hydrophobic (alanine) residue or a small hydrophilic (serine) residue or with large hydrophobic (leucine/isoleucine) residues or large hydrophilic (lysine/glutamine) residues. The tyrosine residue was to be replaced with a hydrophobic (leucine) residue, a hydrophilic (arginine) residue or aromatic (tryptophan/phenylalanine) residues.

3. Methods

3.1 Bacteria Related Methods

3.1.1 Preparation of Competent *E. coli* DH5α

E. coli DH5α cells were inoculated in 3 ml Luria-Bertani (LB) medium with vigorous agitation (250 rpm) at 37 °C over night (ON). A 0.5 ml ON culture was used to inoculate 25 ml fresh LB medium. The cells were grown with vigorous agitation at 37 °C until the optical density at 600 nm (OD_{600}) reached a value of approximately 0.3. The culture was then chilled on ice for 10 min to prevent further growth before the culture was centrifuged at 5000 rpm for 10 min at 4 °C. The supernatant was discarded and the cells were resuspended in 5 ml ice cold 0.1 M $CaCl_2$ and incubated on ice for 5 min. Exposing the cells to calcium ions make them more susceptible for uptake of foreign DNA (*90*). Again, the mixture was centrifuged at 5000 rpm for 10 min at 4 °C. The supernatant was discarded and the cells resuspended in 1 ml 0.1 M $CaCl_2$ containing 15% (v/v) glycerol. The cell suspension was left on ice for approximately 45 min and 100 µl aliquots of competent cells were stored at -80 °C.

3.1.2 Transformation of *E. coli* DH5α

The plasmids (pGEM®-T Easy Vector with insert or the pPlnE100 plasmids) were transformed into *E. coli* DH5α cells according to the QuikChange® site-directed mutagenesis protocol (*91*). The competent *E. coli* DH5α cells were gently thawed on ice and 1 µl of the plasmid DNA (approximately 50 ng/µl) was added to a volume of 50 µl competent cells and immediately transferred on ice for 30 min. The transformation reaction was heat pulsed for 90 seconds (sec) in a microcentrifuge tube at 42 °C for efficient uptake of DNA. The transformation reaction was then incubated on ice for 2 min. A 0.5 ml of LB medium preheated to 42 °C was added to the cell suspension and incubated at 37 °C with vigorous agitation (250 rpm) for up to 1 hour before the suspension was plated on LB agar plates containing the appropriate antibiotic and incubated ON at 37 °C. Depending on the plasmid, the LB agar plates were added either erythromycin (Sigma) to a final concentration of 150 µg/ml (for selection of pPlnE100)

or ampicillin (Calbiochem) to a final concentration of 100 µg/ml (for selection of the pGEM®-T Easy Vector).

3.1.3 Preparation of Competent *Lactobacillus sakei* Lb790/pSAK20

The following procedure was performed as described by Aukrust *et al. (92).* A 25 ml ON culture of *Lactobacillus sakei* (*L. sakei*) Lb790/pSAK20 cells were inoculated in 100 ml de Man-Rogosa-Sharpe (MRS) medium supplemented with glycine to a final concentration of 2% (w/v) and incubated at 30 °C until reaching an OD_{600} of approximately 0.6. Chloramphenicol (Sigma) was added to the growth media (to a final concentration of 10 µg/ml) to ensure that the pSAK20 plasmid was retained in the cells. The supernatant was discarded after the cells were centrifuged for 7 min at 5000 rpm, 4 °C. The cells were resuspended in ice cold 1 mM $MgCl_2$ and again centrifuged for 7 min at 5000 rpm, 4 °C. After another resuspension, this time in 30% ice cold polyethylene glycol (PEG), the cells were centrifuged for 10 min at 7000 rpm, 4 °C, and 100 µl aliquots of competent cells were stored at -80 °C.

3.1.4 Transformation of *L. sakei* Lb790/pSAK20

A volume of 2 µl plasmid DNA (approximately 50 ng/µl) was added to 50 µl competent *L. sakei* Lb790/pSAK20 cells on ice immediately before electroporation. The transformation reaction was carefully transferred to an ice cold electroporation cuvet (2 mm electrode gap) and the electric pulse (making transient holes in the membrane for uptake of DNA) was delivered with the following values:

Voltage: 1.5 kV
Capacitance: 25 µF
Resistance: 400 Ω

The resulting time constants varied between 9.0-9.5.

Immediately after the discharge, 1 ml MRS medium supplemented with 0.5 M sucrose and 0.1 M $MgCl_2$ (MRSSM medium) was added to the electroporation cuvet, gently swirled and transferred to a microcentrifuge tube. The transformation reaction was incubated at 30 °C for 2 h. A volume of 200 µl of the suspension was applied to MRS

agar plates containing 2 µg/ml erythromycin and 5 µg/ml chloramphenicol (Sigma) for selection of pPlnE100 and pSAK20, respectively. The agar plates were incubated at 30 °C for up to 3 days (*92*).

3.1.5 Strains, Cultivation and Storage

Genomic DNA from *L. plantarum* C11 was used as a template in two of the four PCR-reactions performed when creating the insert for construction of the pPlnE100 plasmid (see section 3.5.1 for construction of pPlnE100). The cells were grown in MRS medium at 30 ºC ON without agitation. For long term storage, 850 µl of ON culture was added 150 µl glycerol to a final concentration of 15% and stored at -80 ºC. Glycerol was added to decrease the freezing temperature, thus preventing the cells from lysing.

E. coli DH5α cells were used for amplification and subsequent isolation of the pPlnE100 plasmids and the pGEM®-T Easy Vector (Promega) containing the final PCR product. After transformation, the cells were plated out on LB agar plates containing either a final concentration of 150 µg/ml erythromycin for selection of pPlnE100 plasmids or a final concentration of 100 µg/ml ampicillin for selection of pGEM®-T Easy Vectors with insert. A single colony was added to 3 ml LB containing the appropriate antibiotic and incubated ON with vigorous agitation (250 rpm) at 37 ºC. For long term storage, a 15% glycerol stock was stored at -80 ºC.

L. sakei Lb790/pSAK20/pPlnE100 cells were used for production of wild type and mutant peptides of PlnE. The cells were grown in MRS medium at 30 ºC without agitation. Both chloramphenicol and erythromycin were added to a final concentration of 10 µg/ml for selection of pSAK20 and pPlnE100, respectively.

The indicator strains used in the bacteriocin activity assays were grown in MRS medium or GM17 medium (depending on the strains used) at 30 ºC ON without agitation. For long term storage, the culture was added glycerol as above.

3.2 DNA Related Methods

3.2.1 DNA Isolation from *L. plantarum* C11

The QIAGEN DNeasy® Tissue Kit was used to isolate genomic DNA from *L. plantarum* C11. The isolation was performed according to the protocol for purification of genomic DNA from Gram-positive bacteria (provided by the manufacturer). A total volume of 5 ml ON culture was used to harvest cells.

3.2.2 Plasmid Isolation from *E. coli* DH5α

A Macherey-Nagel NucleoSpin® Plasmid Kit was used to isolate plasmid DNA from *E. coli* according to the protocol specified by the manufacturer. A total volume of 3 ml ON culture was used for purification of plasmids and the elution-step was carried out with 50 µl distilled H_2O (dH_2O).

3.2.3 DNA Purification from Agarose Gel or Solution

The illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare) was used to purify DNA either from an enzymatic reaction or from an agarose gel. Purification was carried out according to the protocol except for the elution step where the isolated DNA was eluted in 30 µl dH_2O instead of the elution buffer supplied by the manufacturer.

3.2.4 Restriction Digests of DNA

The restriction enzymes used were *Cla*I (Promega) and *Mlu*I (Promega). They both recognize and cleave a 6 basepair long palindromic sequence while creating sticky ends on each strand of a dsDNA. For unidirectional cloning, two different restriction enzymes creating sticky ends should be used to ensure correct ligation of insert. After digestion, the two DNA fragments to be ligated together will have overlapping sticky ends. In a subsequent DNA ligation reaction, one end of the insert DNA cleaved by the first restriction enzyme will basepair with the overlapping end of the target DNA sequence while the other end of the insert DNA cleaved by the second restriction

enzyme will basepair with the corresponding overlapping end of the target DNA sequence. An illustration of the DNA fragments applied in this thesis after restriction digestion by *Cla*I and *Mlu*I is shown in Fig 3.1.

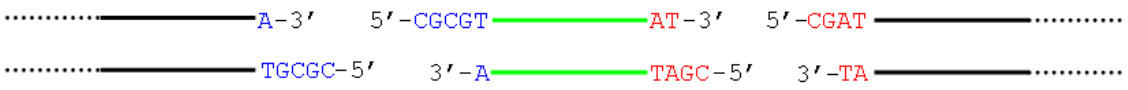


Fig 3.1 Restriction digestion of the insert DNA and vector DNA by *Cla*I and *Mlu*I. Nucleotides marked in blue indicate the sticky ends created by *Mlu*I and the nucleotides marked in red indicate the sticky ends created by *Cla*I. The black lines (dense and stapled) define the vector DNA and the green lines define the insert DNA.

A double restriction digestion with *Cla*I and *Mlu*I was performed with the pGEM®-T Easy Vector with insert and the pLT100β plasmid (a pLPV111 derivative). Buffer C gives a 50-75% of *Mlu*I enzyme activity and a 100% *Cla*I enzyme activity. The restriction digest reactions were carried out according to the protocol provided by the manufacturer (Promega):

5 µl RE 10X Buffer C
0.5 µl of 10 µg/µl acetylated bovine serum albumine (BSA)
0.5-3 µg substrate DNA
1.3 µl *Cla*I (10 U/µl)
1.3 µl *Mlu*I (10 U/µl)
dH_2O to a final volume of 50 µl

The amount of DNA was measured with the Thermo Scientific NanoDrop ND-1000 Spectrophotometer. The reaction mixture was incubated at 37 °C for 2 hours. After complete digestion, the reaction mixture was applied to a 1% agarose gel (section 3.4) for isolation and purification for downstream ligation reaction (section 3.2.6).

3.2.5 *Dpn*I-Treatment of Plasmid DNA

The enzyme *Dpn*I is an endonuclease specific for ^{m6}N methylated adenine in both methylated and hemimethylated DNA with the target sequence 5'-Gm^{6}ATC-3' (*91*). The parental plasmid DNA isolated from *E. coli* DH5α cells contains this target sequence and is therefore susceptible to digestion by *Dpn*I. The mutated plasmid DNA

created by PCR does not contain this methylation and will therefore remain intact after treatment.

Treatment of plasmid DNA with *Dpn*I (Fermentas) was used to digest the parental pPlnE100 plasmid after performing site-directed mutations according to the QuikChange® site-directed mutagenesis protocol (*91*). A volume of 1 µl (10 U/µl) *Dpn*I was added to a 50 µl PCR solution and left for 1 hour at 37 ºC.

3.2.6 DNA Ligation

Ligation of insert into a vector (after digestion with the same restriction enzymes) was performed using a DNA ligase. In this thesis, two ligation reactions were performed. The first reaction involved sub-cloning of the PCR product (the insert) into the pGEM®-T Easy Vector (section 3.3.5) and the second ligation reaction involved ligation of the insert into the pLPV111 plasmid (the pLT100β plasmid becomes a linear pLPV111 plasmid after restriction digestion). Both ligation reactions were performed according to the protocol provided by Promega and Fermentas, respectively:

Ligation of insert into the pGEM®-T Easy Vector:
5 µl 2X Rapid Ligation Buffer (Promega)
1 µl of 50 ng/µl pGEM®-T Easy Vector
1-3 µl PCR product (amount depending on the insert:vector molar ratio used, see below)
1 µl of 3 U/µl T4 DNA Ligase (Promega)
dH_2O to a final volume of 20 µl

Ligation of insert into the pLPV111 plasmid:
2 µl 10X Ligation buffer (Fermentas)
1-9 µl vector DNA (50-400 ng)
1-9 µl insert DNA (amount depending on the insert:vector molar ratio used, see below)
0.5 µl of 10 mM ATP (Amersham Biosciences)
0.5 µl of 5 U/µl T4 DNA Ligase (Fermentas)
dH_2O to a final volume of 20 µl

The insert:vector molar ratios used were 1:1, 3:1 and 5:1. A positive control was performed in the pGEM®-T Easy Vector ligation reactions with the addition of 2 µl (4 ng/µl) Control Insert DNA. For ligation in both pGEM®-T Easy Vector and the

pLPV111 plasmid, a negative control was performed without the insert DNA to check for self-ligation of the plasmid. The ligation reactions were incubated at 4 °C ON.

3.2.7 DNA Sequencing

A sequencing sample was prepared and delivered to our sequence facility which uses the BigDye® Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM® 3730 DNA Analyzer for DNA sequencing.

The sequencing sample:
4-8 µl DNA (150-300 ng of plasmid DNA or 10-40 ng of PCR product)
0.5 µl of a 20 µM sequencing primer
dH_2O to a final volume of 10 µl

An overview of all sequencing primers used in this study is listed in the Appendix, section 6.4.1.

3.3 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a simple method for amplifying specific DNA fragments on a defined DNA sequence. It is often used to introduce additional sequences such as restriction sites or the introduction of site-directed mutations in the original DNA sequence (*93*). For each cycle of amplification performed, the amount of target DNA currently present is doubled (*94*). A PCR reaction typically consists of 16-30 cycles. When exceeding this number, the amount of newly synthesized product is limited by the amount of primers present (*94*). The main components in the reaction-mixture are: a thermo stabile DNA polymerase, two oligodeoxynucleotide (oligo) primers (forward and reverse), a DNA template, the four deoxynucleoside triphosphates (dNTPs; dATP, dTTP, dGTP and dCTP) and an optimal buffer for the DNA polymerase. Each cycle includes three steps: denaturation, annealing and 3' extension. In the first step, the dsDNA is heat denatured into single stranded DNA (ssDNA). In the second step, the temperature is lowered to make sure the primers anneal to their complementary region in the target DNA sequence. The thermo stabile DNA polymerase then synthesizes the complementary DNA strand in the third and last step by adding dNTPs to the 3' end of both primers (forward and reverse) and thereby

completing the synthesis of the desired DNA sequence. When the thermo-cycling is done, an extra 3' extension-step is performed to ensure complete elongation of the final PCR product.

Three different DNA polymerases have been used in this study: *Pfu* DNA polymerase (Fermentas), *PfuTurbo* DNA polymerase (Stratagene) and *Taq* DNA polymerase (Fermentas). *Taq* DNA polymerase was used when it was necessary to make dATP-overhangs in each 3'-end of the PCR product to facilitate ligation into a pGEM®-T Easy Vector System. All the PCR reactions applied in this thesis were carried out using a MJ Research PTC-200 Peltier Thermal Cycler. The different primers used for creation of insert and site-directed mutagenesis were ordered from Eurogentec S.A. and are listed in the Appendix, section 6.4.

3.3.1 Gradient PCR

Gradient PCR is a useful way to test for optimal annealing temperatures for primers. This can help minimize the effect of unspecific primer-annealing by increasing the specificity, thus creating fewer non-specific extension-products leading to increased quantity of the targeted DNA fragment (*94*).The temperatures were set to span between 48 ºC and 59 ºC and the value which gave the strongest DNA-band after gel electrophoresis was set as default in the subsequent PCR programs.

3.3.2 The PCR Megaprimer Method

The megaprimer method can be used for site-directed mutagenesis (*95*) as well as making a fusion-product out of two different DNA-sequences. In general, this method utilizes three oligonucleotide primers and two rounds of multiple cycles of PCR to create the desired PCR product. In the first reaction, two oligonucleotide primers anneal to their complementary DNA fragment for amplification. One of the primers contains a tail complementary to the DNA to be amplified in the second PCR. The resulting PCR product from the first PCR reaction can then be used as a megaprimer in the second round of PCR. Together with a third oligonucleotide primer, a completely new DNA sequence may be synthesized. An important distinction between the PCR megaprimer

method and the regular PCR method is that the megaprimer, in addition to partial overlap of the second template DNA, also has a tail at one end comprising the first PCR-product. The megaprimer thereby unites two different DNA sequences, making a longer DNA sequence than both the precursors.

Parameters for the Megaprimer Method

The megaprimer method was used to make a fusion-product of the sakacin A promoter, the sakacin P leader sequence and the *plnE*-gene encoding the mature PlnE peptide as well as coupling the beginning of the *plnI*-gene (encoding the cognate immunity protein) to this sequence. In the first round of PCR, the template DNA was the pLT100β plasmid (Appendix, section 6.6), created for another two-peptide bacteriocin construct. The two primers SakPB and PlnEA (both from Eurogentec S.A.) were used to amplify the gene encoding the sakacin P leader sequence as well as the sakacin A promoter and a restriction site for *Mlu*I positioned upstream of the promoter region. The PlnEA primer contains a tail complementary to the beginning of the *plnE*-gene. The resulting PCR product can be used as a megaprimer in the second round of PCR; hence it was termed Megaprimer 1 (Fig 3.2, round 1). In the next round of PCR, the template DNA was genomic DNA from *L. plantarum* C11, which contains the *plnEFI*-operon. Megaprimer 1 and the PlnEC primer (Eurogentec S.A.) were used to amplify the *plnE*-gene. In addition, the PlnEC primer contains a tail containing the beginning of the *plnI*-gene. The product of the second round of PCR, named Megaprimer 2, will now consist of the sakacin A promoter, the *plnE*-gene fused to the gene encoding the sakacin P leader sequence and the beginning of the *plnI*-gene (Fig 3.2, round 2).

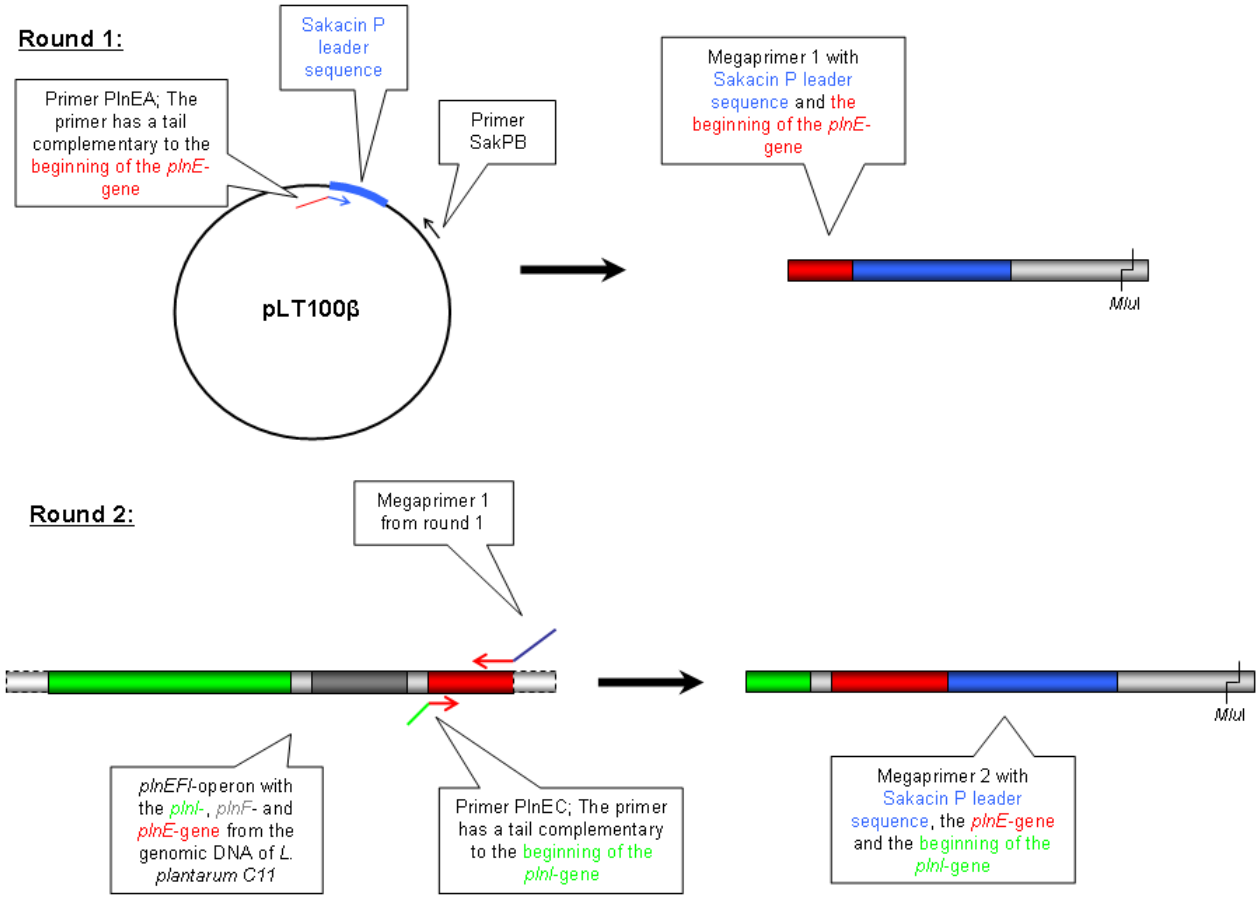


Fig 3.2 The megaprimer method illustrating the first two rounds of PCR. Round 1: The sakacin P leader sequence, the sakacin A promoter region and the restriction site for *Mlu*I from the pLT100β plasmid are fused together with the beginning of the *plnE*-gene by the primers SakPB and PlnEA, creating Megaprimer 1 for the second round of PCR. The grey box to the right in Megaprimer 1 and Megaprimer 2 indicates the sakacin A promoter region with a restriction site for *Mlu*I. Round 2: Megaprimer 1 and the primer PlnEC anneals to each end of the *plnE*-gene from the genomic DNA of *L. plantarum* C11, having a tail comprising the sakacin P leader sequence and the beginning of the *plnI*-gene, respectively. The product now consists of the sakacin A promoter, the sakacin P leader sequence, the *plnE*-gene and the beginning of the *plnI*-gene as well as the restriction site for *Mlu*I, denoted Megaprimer 2 as for the next PCR reaction.

The reaction conditions for the first and second PCR reactions were carried out according to the protocol provided by the manufacturer (Fermentas):

Round 1:
5 µl *Pfu* Buffer (without $MgSO_4$)
6 µl of 25 mM $MgSO_4$
8 µl of 5 mM dNTP mix (1.25 mM each; Pharmacia Biotech)
1-4 µl pLT100β plasmid DNA (50 – 100 ng)
5 µl of 20 µM SakPB
5 µl of 20 µM PlnEA
0.5 µl of 2.5 U/µl *Pfu* DNA polymerase
dH_2O to a final volume of 50 µl

Round 2:
5 µl *Pfu* Buffer (without $MgSO_4$)
6 µl of 25 mM $MgSO_4$
8 µl of 5 mM dNTP mix (1.25 mM each)
2 µl genomic DNA of *L. plantarum* C11 (0.1 – 1 µg)
5 µl of 20 µM PlnEC
5-10 µl Megaprimer 1 (230 ng)
0.5 µl of 2.5 U/µl *Pfu* DNA polymerase
dH_2O to a final volume of 50 µl

After an initial heat shock for degradation of interfering enzymes, *Pfu* DNA polymerase was added to the mixture. The cycling parameters are listed in Tables 2.1 and 2.2.

Table 2.1 Cycling parameters for the PCR megaprimer method, round 1:

Step	Temperature	Time	Cycle
Heat shock	95 ºC	2 min	1
Denaturing	95 ºC	1 min	25
Annealing	48 ºC	1 min	
Extension	72 ºC	2 min (2 min/kb)	
Extension	72 ºC	7 min	1

Table 2.2 Cycling parameters for the PCR megaprimer method, round 2:

Step	Temperature	Time	Cycle
Heat shock	95 ºC	10 min	1
Denaturing	95 ºC	45 sek	25
Annealing	55 ºC	1 min	
Extension	72 ºC	2 min (2 min/kb)	
Extension	72 ºC	7 min	1

3.3.3 PCR Splicing by Overlap Extension (PCR SOEing)

PCR SOEing is a simple way of fusing two DNA fragments together, creating mutations and/or deletions as well the generation of recombinant DNA products (*93*). Generally, it involves two independent PCR reactions and one third, "splicing" reaction. The first two sample reactions contain both one external and one internal primer. The internal primer contains the mutation of interest. The internal primers are complementary to each other, thereby enabling the two products to overlap. Both the external and internal primers anneal at each 3' end of the target DNA sequence. The third PCR reaction involves splicing by elongation of the two overlapping PCR products. In the first cycles of this last round of PCR, the two PCR products are added to the reaction mixture. As they overlap at each 3' end, they will act as primers for each other; hence the DNA polymerase will extend the hybridized fragments from each 3' end, making the final PCR product. When the PCR product is synthesized, two external oligo primers are added to the reaction mixture, each complementary to the 3' end of the two PCR products from the first two rounds of PCR, thereby letting the DNA polymerase amplifying the final product (*93*).

Parameters for PCR SOEing

The PCR SOEing method was used to fuse the *plnI*-gene together with the resulting fragment (termed Megaprimer 2) from the megaprimer method by overlap extension, thereby creating the final PCR product (the insert). The template DNA in the first round of the PCR SOEing method was genomic DNA of *L. plantarum* C11. The two primers added were PlnEFimmstart and PlnEFimm (both from Eurogentec S.A.).

PlnEFimmstart contains a tail complementary to the *plnE*-gene and PlnEFimm contains a point mutation making a restriction site for *Cla*I, positioned downstream of the *plnI*-gene for later insertion into an expression vector. The DNA polymerase then amplifies the *plnI*-gene (Fig 3.3). This intermediary PCR product and Megaprimer 2 were spliced together in the next step (Fig 3.4). The spliced PCR product was amplified by adding the two external primers, primer PlnEFimm and SakPB (Fig 3.4). The final PCR product now consists of the entire *plnI*-gene, the *plnE*-gene fused to the gene encoding the leader sequence of sakacin P and the sakacin A promoter region. The restriction site for *Cla*I is positioned after the *plnI*-gene, and the restriction site for *Mlu*I is positioned in front of the promoter region of sakacin A.

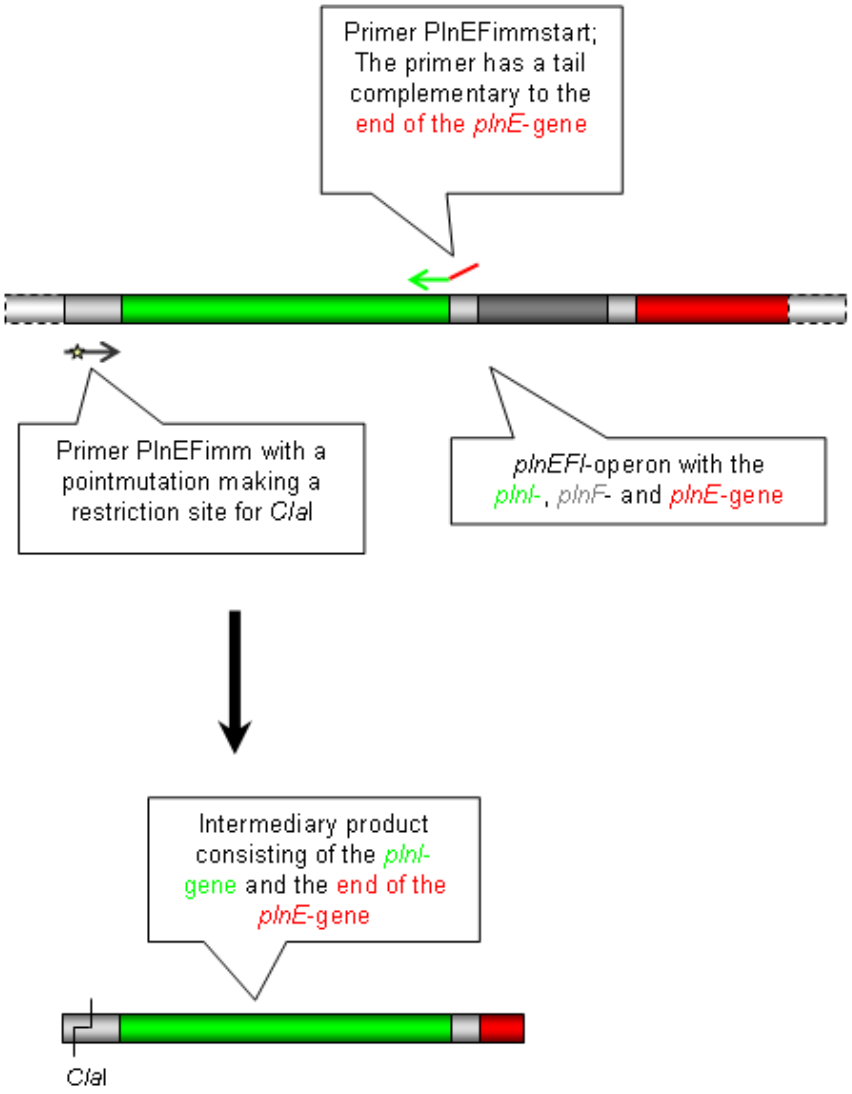


Fig 3.3 The PCR SOEing method illustrating the first PCR SOEing reaction. Primer PlnEFimm contains a point mutation making a restriction site for *Cla*I at the downstream region of the *plnI*-gene when extended. PlnEFimmstart anneals to the beginning of the *plnI*-gene while at the same time carrying a tale comprising the end of the *plnE*-gene, making an intermediary product which will overlap with Megaprimer 2 in the final PCR reaction (Fig 3.4).

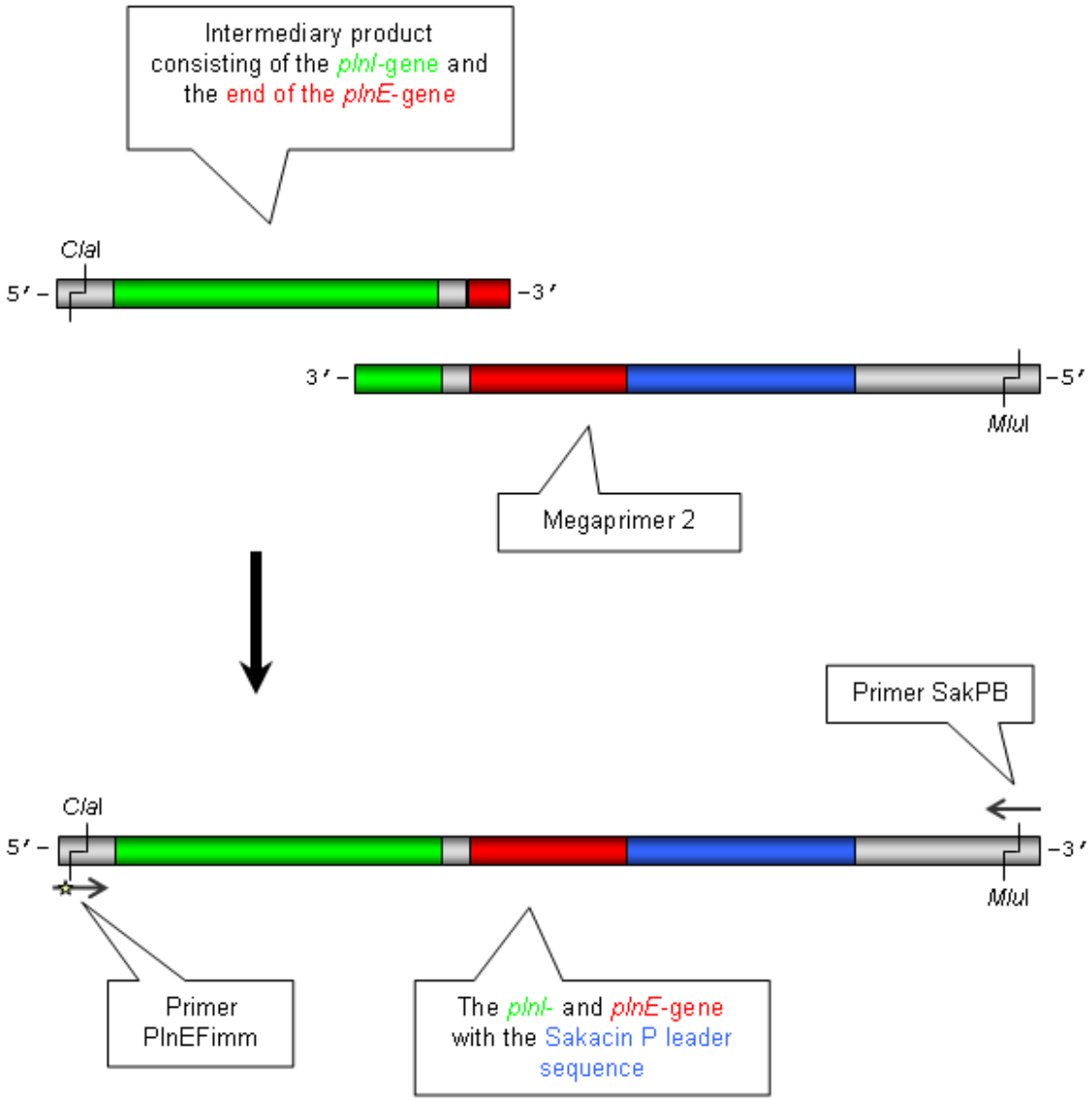


Fig 3.4 The PCR SOEing method illustrating the second PCR SOEing reaction. The intermediary product overlaps with Megaprimer 2, acting as primers for each other when the DNA polymerase extends the two sequences from each 3' end. For amplification of the final PCR product, the two primers PlnEFimm and SakPB are added in the last cycles of the final PCR reaction. The grey box to the right in Megaprimer 2 and the final PCR product indicates the sakacin A promoter region with a restriction site for *Mlu*I.

The reaction conditions were carried out according to the manufacturer (Fermentas):

1. PCR reaction of PCR SOEing:
5 µl *Pfu* Buffer (without $MgSO_4$)
6 µl of 25 mM $MgSO_4$
8 µl of 5 mM dNTP mix (1.25 mM each)
2 µl genomic DNA of *L. plantarum* C11 (0.1 – 1 µg)
2.5 µl of 20 µM PlnEFimm
2.5 µl of 20 µM PlnEFimmstart
0.5 µl of 2.5 U/µl *Pfu* DNA polymerase
dH_2O to a final volume of 50 µl

2. PCR reaction of PCR SOEing:
5 µl *Pfu* Buffer (without $MgSO_4$)
6 µl of 25 mM $MgSO_4$
8 µl of 5 mM dNTP mix (1.25 mM each)
210 ng intermediary PCR product
210 ng Megaprimer 2
5 µl of 20 µM SakPB
2.5 µl of 20 µM PlnEFimm
0.5 µl of 2.5 U/µl *Pfu* DNA polymerase
dH_2O to a final volume of 50 µl

After an initial heat shock for degradation of interfering enzymes, *Pfu* DNA polymerase was added to the mixture in both PCR reactions. After 10 cycles of PCR in the second PCR reaction the primers SakPB and PlnEFimm were added for amplification of the final PCR product. Cycling parameters for both reactions are listed in Table 2.3.

Table 2.3 Cycling parameters for the PCR SOEing method:

Step	Temperature	Time	Cycle
Heat shock	95 ºC	10 min	1
Denaturing	95 ºC	45 sek	25 [a)]/10[b)]/25[c)]
Annealing	48 ºC	1 min	
Extension	72 ºC	3 min (2 min/kb)	
Extension	72 ºC	7 min	1

[a)] The first PCR reaction (creating the intermediary product) was performed with 25 cycles.
[b)] The first step of the second PCR reaction (for splicing of the two DNA fragments) was performed with 10 cycles.
[c)] The final step of the second PCR reaction (for amplification of the final PCR product) was performed with 25 cycles.

3.3.4 QuikChange® Site-Directed Mutagenesis

QuikChange® site-directed mutagenesis is a technique which can be used for the creation of point mutations, switching of amino acids and deletion/insertion of single or multiple amino acids. It is a rapid four-step procedure (Fig 3.5) that generates specific mutated plasmids with greater than 80% efficiency (*91*).

The protocol includes the use of a double stranded plasmid DNA, two mutagenic oligo primers and the *PfuTurbo* DNA polymerase (Stratagene). The primers are complementary to each other and the plasmid DNA to be amplified. After initial denaturation of the plasmid DNA and subsequent annealing of primers, *PfuTurbo* DNA polymerase elongates the mutated plasmid DNA, leaving the PCR product with staggered nicks. The product is then treated with the endonuclease *Dpn*I (section 3.2.5) which digests the parental plasmid DNA, leaving only the nicked plasmids containing the desired mutation. The fourth and last step of this procedure is to transform the nicked plasmids into competent *E. coli* DH5α cells, which will then repair the nicks (*91*).

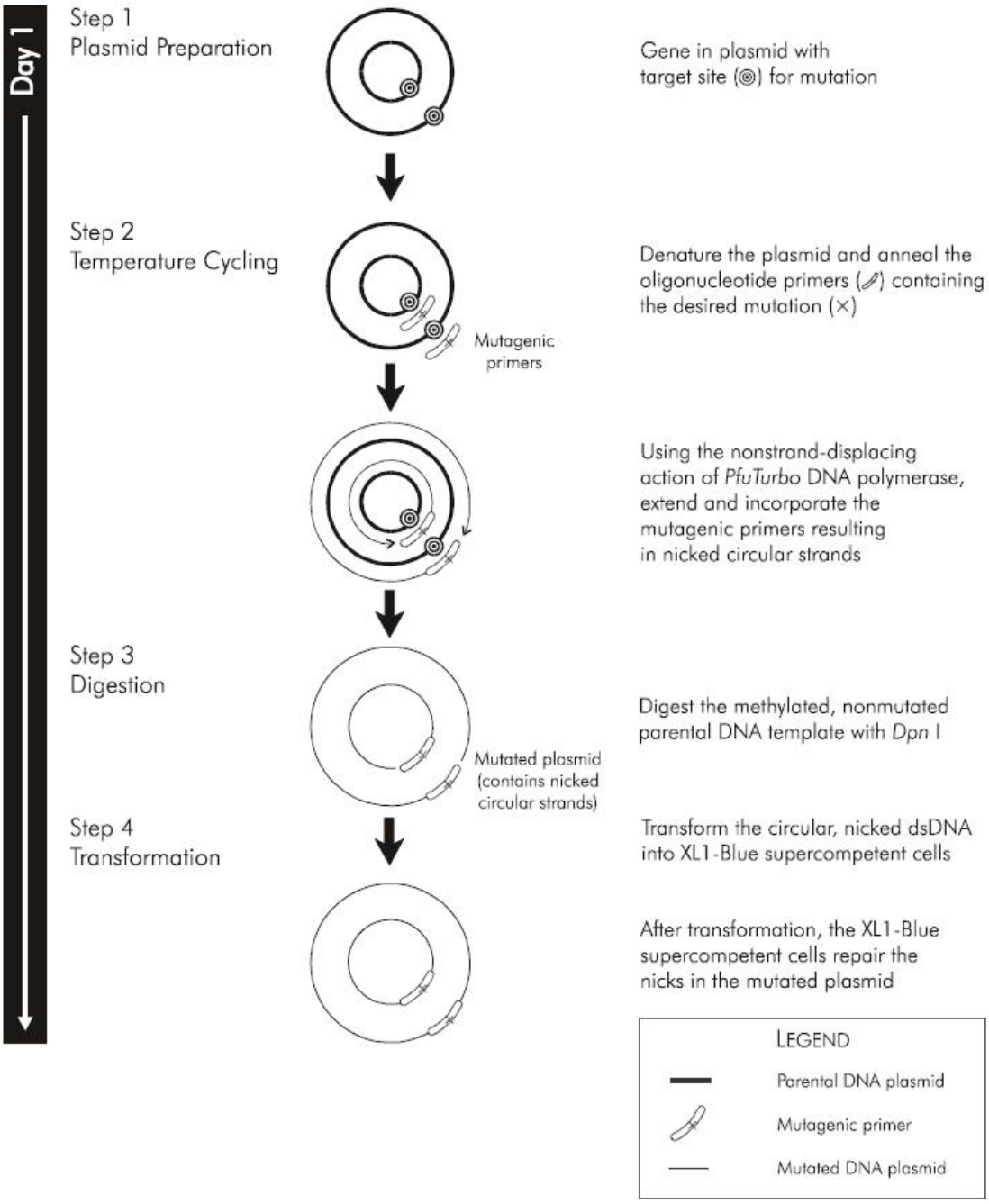


Fig 3.5 The four-step procedure for QuikChange® site-directed mutagenesis. Step 1: A plasmid DNA containing the gene with the target site (◎) is constructed. Step 2: The plasmid is denatured by thermal cycling and the two synthetic oligonucleotide primers containing the desired mutation anneal to each strand of the template DNA at the site of mutation. The *PfuTurbo* DNA polymerase extends and incorporates the mutagenic primers, creating nicked DNA plasmids. Step 3: The nonmutated, methylated parental strand is digested by treatment with *Dpn*I. Step 4: The nicked, mutated plasmid is repaired when transformed into competent *E. coli* DH5α cells. The figure is adapted from QuikChange® site-directed mutagenesis protocol (*91*).

Parameters for Quik Change Site-Directed Mutagenesis

In this thesis, the method was used to introduce a specific point mutation leading to single amino acid changes in the *plnE*-gene. Each mutation was constructed by the use of specific primers, a forward (F) primer and a reverse (R) primer annealing each strand of the plasmid DNA (pPlnE100) at the desired site of mutation. The site-directed mutations were performed basically according to the instruction manual from the QuikChange® site-directed mutagenesis kit (*91*). All the primer sequences used for the introduction of the desired mutations into the *plnE*-gene are listed in the Appendix, section 6.4.2. The primers were ordered from Eurogentec S.A. The reaction conditions were carried out according to the QuikChange® mutagenesis protocol (Stratagene):

The PCR mixture:
5 µl 10X Cloned Pfu Reaction Buffer
1 µl plasmid DNA (5 – 50 ng)
1.3 µl forward primer (130 ng)
1.3 µl reverse primer (130 ng)
2 µl of 5 mM dNTP-mix (1.25 mM each)
1 µl of 2.5 U/µl *PfuTurbo* DNA polymerase
38.4 µl dH_2O to a final volume of 50 µl

After an initial heat shock, *PfuTurbo* DNA polymerase was added to the mixture.

3.3.5 The pGEM®-T Easy Vector System

The pGEM®-T Easy Vector System can be used for cloning of a PCR product. The vector is prepared by cutting with the restriction enzyme *Eco*RV and subsequent addition of 3' terminal thymidine to both ends. The PCR product to be inserted is likewise treated with a thermostable DNA Polymerase which adds a 3' terminal deoxyadenosine in a template-independent fashion in both ends. This prevents recirculization of the vector in a subsequent ligation reaction as well as providing an overlapping overhang between the PCR product and the vector. The vector also contains a multiple cloning site (MCS) as well as a gene encoding the enzyme β-galactosidase positioned within this region. This allows for blue-white screening of recombinant clones (*96*). A circle map of the pGEM®-T Easy Vector is listed in the Appendix, section 6.6.

Parameters for the pGEM®-T Easy Vector Cloning

The pGEM®-T Easy Vector System was put to use for sub-cloning of the PCR product (the insert). Addition of a 3' terminal deoxyadenosine to both ends of the insert was performed with the *Taq* DNA Polymerase (Fermentas) in a thermal cycler:

Addition of a single 3' terminal deoxyadenosine overhang:
1 µl 10X *Taq* Buffer with $(NH_4)_2SO_4$
1 µl of 25 mM $MgCl_2$
1 µl of 2 mM dATP (Pharmacia Biotech)
6 µl PCR product
1 µl of 5 U/µl *Taq* DNA Polymerase
10 µl total

The reaction was incubated at 70 ºC for 30 min. See section 3.2.3 for purification of the enzymatic reaction.

3.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis is a method used to separate DNA and RNA molecules by size. Agarose is a polysaccharide which will form a porous matrix upon heating when added to an electrophoresis buffer. An electrical field is applied to the gel and the negatively charged DNA will be sieved through the pores towards the anode. The velocity of the DNA molecule is proportional to its size, thus the shorter the lengths of a DNA fragment, the greater is its elecrophoretic mobility (*97*). For plasmids, the migration rate is as follows: Supercoiled plasmid > circular plasmid > linearized plasmid. Different factors affecting the migration rate of the DNA molecule have to be taken into consideration when performing agarose gel electrophoresis: the concentration of the agarose, the voltage applied and the composition and ionic strength of the buffer. A high concentration of agarose yields smaller pores; hence DNA will spend more time wandering toward the anode than in larger pores. At high electrical field strengths, the mobility of the DNA molecule will increase, but this may as well decrease the resolution of the separation. At low ionic strength of the buffer, the DNA will migrate slowly because of the low electrical conductivity (*97*). When preparing the gel, the solution is heated up to its boiling point in order to dissolve the agarose. After cooling, ethidium bromide (EtBr) is added to the liquid gel-solution. EtBr is an intercalating

agent which enters the space between the bases in the DNA double helix and functions as a fluorescent dye. The DNA can then be visualized under ultraviolet (UV) light which can reveal distinct band patterns.

The plasmid DNA, the restricted DNA fragments and the PCR products were analyzed, and where appropriate, excised from the agarose gel. A 1% (w/v) agarose solution was made by dissolving 0.5 g agarose (Merck) in 50 ml Tris-acetate-EDTA (TAE) buffer by heating in a microwave oven. After cooling to 60 °C (to make sure no degradation of EtBr will occur), EtBr was added before casting the gel. The agarose gel electrophoresis was usually run for 45 min at 70 volts (V) and with an electrical current of 80 milli ampere (mA). The DNA ladders used for sizing were the 100 bp DNA Ladder, 100 bp DNA Ladder Plus or the 1 kb DNA Ladder (all manufactured by Fermentas). The DNA ladders are shown in the Appendix, section 6.5.

3.5 Protein Related Methods

3.5.1 Expression of Wild Type and Peptide Variants of PlnE

A heterologous expression system of class-II bacteriocins in a bacteriocin-deficient host strain, producing bacteriocins at levels equal to or higher than the corresponding wild type strain has been described (*98*). This may help determine the effect of different bacteriocins in food, to construct new LAB strains with improved protective properties and to improve the efficiency of expression systems for structure-function analysis by *in vitro* site-directed mutagenesis (*98*). The system is based on the introduction of two plasmids into the bacteriocin-deficient strain *L. sakei* Lb790 and the expression of a class-IIa bacteriocin, sakacin A. The genes necessary for production of sakacin A are located on two divergently transcribed operons and have been shown to complement each other when separated on two plasmids (*99*). One operon contains the gene encoding sakacin A, *sapA*, and its cognate immunity protein, encoded by the gene *saiA*. The other operon, *orf4-sapKRTE*, encodes the three-component regulatory unit responsible for the control of bacteriocin production and synthesis of proteins involved in bacteriocin secretion. The genes encoding the regulatory unit encompasses *orf4* (encodes a peptide pheromone (*100*)), *sapK* (encodes a histidine protein kinase) and

sapR (encodes a response regulator) (*99*). The genes encoding the proteins involved in secretion are *sapT* (encodes an ABC transporter) and *sapE* (encodes an accessory factor) (*99*). The *orf4-sapKRTE*-operon is located on the pSAK20 plasmid along with a marker for chloramphenicol-resistance (*cat*) (*99*). The second plasmid, a pLPV111 derivative, contains the structural and immunity genes of the bacteriocin of interest fused to the sakacin A promoter as well as a marker for erythromycin-resistance (*ermL*) (*98*). The plasmid pLPV111 and its derivatives are shuttle-vectors that can be replicated in both *E. coli* and *L. sakei*. All plasmids used in this study are shown in the Appendix, section 6.6.

The heterologous expression system was used for the production of wild type and peptide variants of PlnE. The cognate immunity protein, PlnI, was co-expressed for protection of the producer strain. Also, a previous study has implicated that a slight increase in bacteriocin-production is seen when the immunity-gene is expressed together with the bacteriocin (*101*).

A pLPV111 derivative, named pPlnE100, containing the structural (*plnE*) and immunity gene (*plnI*) of PlnE fused to the sakacin P leader sequence and the sakacin A promoter was constructed. The operon *plnEI* is now placed under control of the sakacin A promoter. Previous studies have demonstrated that the sakacin A secretion machinery encoded in pSAK20 recognizes both the sakacin A and sakacin P leader peptides equally efficient (*98*). The plasmid pPlnE100 was constructed from another pLPV111 derivative, named pLT100β. The latter plasmid carries the sakacin A promoter, the gene encoding LcnG-β (one of the peptides constituting the two-peptide bacteriocin lactococcin G) fused to the sakacin P leader sequence, as well as the gene encoding the lactococcin G immunity protein. It also contains a marker for erythromycin-resistance (*ermL*). The pPlnE100 plasmid was constructed by digesting the insert as well as the pLT100β plasmid with the restriction enzymes *Cla*I and *Mlu*I (section 3.2.4), creating a linearized pLPV111 plasmid. After digestion, the insert was ligated into the vector.

3.5.2 Chromatographic Separation of Proteins

Chromatography is a common method for purification of proteins and for analysis of proteins in a sample. The principle of the separation of a protein in chromatography is roughly based upon a system of two phases, in biochemistry normally being either liquid-liquid or liquid-solid (*102*). The stationary phase is attached to the column while the mobile phase (termed eluent when applied to the column and eluate when eluting from the column along with the components) is a free flow of liquid passing through the stationary phase. In a liquid-liquid system, the proteins may or may not partition themselves between the two phases depending on their solubility. In a liquid-solid system, the proteins can adsorb at varying degrees to the solid phase. In either scenario, the concentration ratio of a protein can be expressed as the partition coefficient, K

$$K = \frac{C_S}{C_M} \qquad \text{(eq. 2.1)}$$

where C_S and C_M are the sample concentrations in the stationary and mobile phases, respectively (*102*). Two disparate proteins will have different partition coefficients, thus being retained on the column at varying degrees and therefore also migrate through the column at different velocities (different retention times).

A rapid two-step procedure for purification of pediocin-like bacteriocins and other cationic antimicrobial peptides has been described by Uteng *et al.* (*103*). The method is based on the cationic and hydrophobic properties of antimicrobial peptides. The first purification step relies on the passage of bacterial cultures through a cation-exchange column and subsequent elution with 1 M NaCl, whereas the second purification step removes remaining contaminants by the use of reverse-phase chromatography upon elution with propanol. Isolation and purification of wild type and peptide variants of PlnE was performed basically according to the procedure described by Uteng *et al. (103)*, but with the addition of 2-propanol (at a final concentration of 20% (v/v)) to the elution buffer in cation-exchange chromatography in order to obtain a higher yield of peptides in this step (Oppegård, C., personal communication).

3.5.3 Cation-Exchange Chromatography

In biochemistry, ion-exchange chromatography is a method for separating water-soluble proteins (as well as DNA) in a solution by taking advantage of their different surface charges at a given pH. Knowledge about a proteins surface charge and its isoelectric point (pI) can be used to separate it from other components in a sample. Ion-exchange chromatography is a liquid-solid system, thus the proteins will adsorb to the stationary phase based on the attraction between the net charge of the protein and the charged groups attached to the stationary phase. The solid support (the stationary phase) is made up of polymers such as cellulose and agarose. The ion exchangers are covalently bonded to the solid support and are either negatively or positively charged. An anion-exchanger has covalently bound cations and exchangeable anions, whereas a cation-exchanger has covalently bound anions and exchangeable cations (*102*). In cation-exchange chromatography, a protein with a net positive charge will adsorb to the negatively charged functional groups, while a protein with net negative charge or no net charge will pass through the column in conjunction with the mobile phase. When eluting the proteins, the column is conditioned with a large excess of salt, thus exchanging the adsorbed protein with a counterion. Alternatively, the pH of the mobile phase can be altered in the direction to which the protein changes its net charge to the opposite, resulting in a very weak or non-existent attraction between the functional groups and the protein.

Parameters for Cation-Exchange Chromatography

The peptides PlnE and PlnF are both positively charged at neutral pH and are therefore good candidates for separation by cation-exchange chromatography. A cation-exchanger SP-Sepharose Fastflow column from GE Healthcare was used for the first purification step. The material consisted of 6% crosslinked agarose with sulphopropyl (SP) groups covalently bound to it and the column had a volume of 100 ml. A 10 ml ON culture was applied to 1 liter (L) of MRS medium containing erythromycin and chlormaphenicol to a final concentration of 10 µg/ml for selection of the plasmids pPlnE100 and pSAK20, respectively. The cells were grown to stationary phase and were then directly applied to the cation-exchange column after equilibration with a 20 mM phosphate buffer (pH 6). After 1 L of cell culture had been poured through the

column (approximately 10 column volumes), the column was washed with 1 column volume of the phosphate buffer. The peptides were eluted with 40 ml of a 1 M NaCl, 20 mM phosphate buffer containing 20% (v/v) 2-propanol (pH 5.5). The eluate was sterile-filtrated through a 0.20 µm non-pyrogenic sterile filter (Sarstedt).

3.5.4 Reverse-Phase Chromatography

In reverse-phase chromatography, proteins and peptides are separated by their differences in net hydrophobicity. The separation is based on a liquid-liquid partition system where the support is made up of microporous particles of silica and the stationary phase is covalently attached to the polymer (*104*). In biochemistry, the stationary phase generally consists of a non-polar hydrocarbon chain and the mobile phase is more polar than the stationary phase. The proteins will partition themselves between the stationary and mobile phases based on hydrophobic interactions to the former (*102*). When performing gradient elution, the eluent strength will increase as the mobile phase becomes less polar. The peptides with low net hydrophobicity are the ones with the shortest retention times and the peptides with the highest net hydrophobicity will have the longest retention times, hence elute closer to the end of the gradient step.

In this study, reverse-phase chromatography using the ÄKTA system (Amersham Biosciences) was used as the second purification step for removing remaining contaminants in the peptide solution.

Parameters for Reverse-Phase Chromatography

All of the eluate (40 ml) obtained from the cation-exchange column was diluted by a factor of four with dH_2O/0.1% (v/v) trifluoroacetic acid (TFA) and applied to the reverse-phase column (3 ml RESOURCE™ RPC, GE Healthcare) consisting of polystyrene/divinylbenzene. The elution step was performed using a linear, segmented elution gradient divided into three segments. The first segment started at 0% (v/v) 2-propanol/0.1% (v/v) TFA and ended at 20% (v/v) 2-propanol/0.1% (v/v) TFA. The volume of eluent was set to 3 ml (1 column volume). The second segment started at 20% (v/v) 2-propanol/0.1% (v/v) TFA and ended at 60% (v/v) 2-propanol/0.1% (v/v) TFA and the volume of eluent was set to 30 ml (10 column volumes). The third segment started at 60% (v/v) 2-propanol/0.1% TFA and ended at 100% (v/v) 2-propanol/0.1%

TFA. Again, the volume of eluent was set to 3 ml (1 column volume). The solution containing dH_2O/0.1% (v/v) TFA had a pH of approximately 2 and the solution containing 2-propanol/0.1% (v/v) TFA had a pH of approximately 4. The flow rate of the mobile phase was 2 ml/min, and the absorbance at 280 nm and 214 nm was recorded as a function of ml eluent.

3.5.5 Analyzing the Degree of Purity

The degree of purity from the fractions containing the wild type and mutant peptides of PlnE was analyzed by analytical reverse-phase chromatography using the SMART system (Amersham Biosciences). The column used was a µRPC C_2/C_{18} Sc.2.1/10 (Amersham Biosciences) and the gradient was set to range from 0% (v/v) 2-propanol/0.1% (v/v) TFA to 50% (v/v) 2-propanol/0.1% (v/v) TFA. The volume of eluent was 2 ml. The mobile phase flow rate was 100 µl/min and the absorbance at 214 nm was recorded as a function of ml eluent.

3.5.6 Matrix Assisted Laser Desorption Ionisation Time-of-Flight (MALDI-TOF)

In general, mass spectrometry (MS) is a method for analysis of components by measuring their mass/charge (m/z) ratios by the formation of molecular ions in a sample. It consists of an ionisation source, an analyser and a detector. The size of the molecules defines the amount of deflection in a magnetic field. A large ion will be deflected to a lesser extent than a smaller ion with identical charge (*102*). Matrix-assisted laser desorption ionisation (MALDI) is a method for molecular weight determination of proteins and peptides in a sample. The sample and a suitable matrix is bombarded and vaporised in a jet with pulses of high-energy UV radiation. The matrix absorbs most of the energy, enabling the sample components to stay intact during radiation. The sample molecules are ionised by the excited matrix molecules, creating predominantly positively single-charged sample molecules, but multi-charged molecules may also be formed (*102*). MALDI can be combined with a time-of-flight (TOF) analyser where the ions are accelerated down a drift tube without a magnetic field (Fig 3.6). The ions will have the same kinetic energy, meaning that the velocity (and their TOF) will only depend on their molecular masses. The amount of time each

ion spends travelling through the drift tube toward the detector is measured and the m/z value is calculated (*102*).

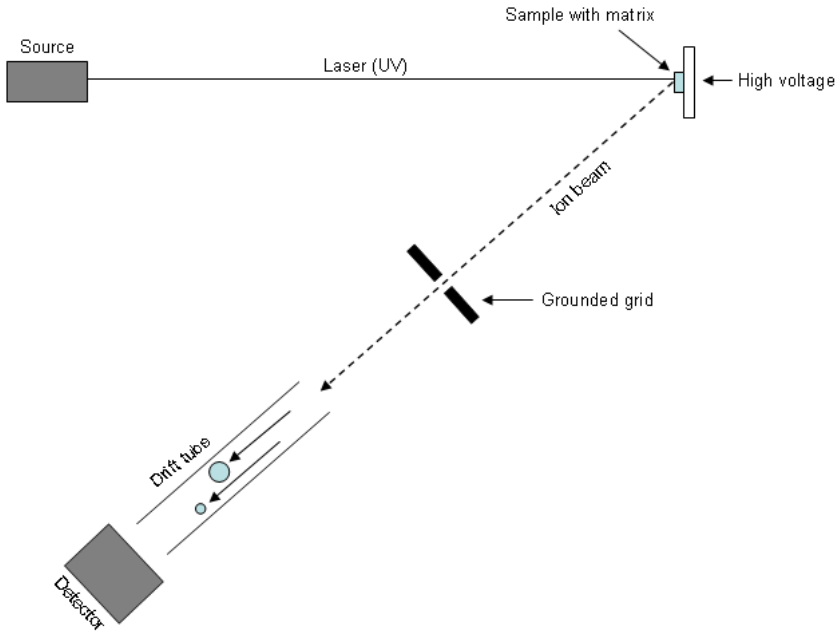


Fig 3.6 Schematic overview of MALDI-TOF MS. The sample and matrix is bombarded and vaporised with a pulse of UV radiation, thereby creating positively charged sample molecules (MH^+) by the excited matrix molecules. The sample ions are accelerated down the drift tube so that they can be separated based on their time-of-flight and their m/z ratios can be calculated.

A volume of 10 µl of each sample was delivered to MS/Proteomics Core Facility at the Department of Chemistry and Biotechnology, Norwegian University of Life Sciences, Ås.

3.5.7 Estimation of Peptide Concentrations

PlnE and most of the peptide variants of PlnE contain only one residue (a tyrosine residue) with absorbance at 280 nm and the extinction coefficient of the peptides at 280 nm is consequently relatively low. The concentration of the purified peptides obtained after reverse-phase chromatography was too low to quantitate the amount of peptides by measuring the absorbance at this wavelength. As a consequence, the absorption peaks obtained at 214 nm upon reverse-phase chromatography were used to quantitate the peptides (i.e. by measuring the peak areas and subtracting estimated background noise). The extinction coefficient of the peptides at 214 nm was estimated to be approximately 50000 $M^{-1}cm^{-1}$, calculated from the contributions of the individual amino acid residues at 214 nm and the peptide bonds (*105*).

3.5.8 Bacteriocin Activity Assay

For detection of antimicrobial activity of the wild type and peptide variants of PlnE, a microtiter plate assay system was used, essentially as described by Nissen-Meyer *et al.* (*26*). The growth of the indicator cells was measured spectrophotometrically at 600 nm by use of a MR700 Microplate Reader from Dynatech. Each well of the microtiter plate contained M17 medium supplemented with 0.4% (wt/v) glucose and 0.1% (v/v) Tween 80 (GM17 medium) or MRS medium, the mutated or wild type PlnE peptide, the wild type PlnF peptide, and the indicator strain, all to a final volume of 200 µl. The dilution factor of the peptide combinations was twofold going from one well to the next. Stationary phase cultures of indicator strains were diluted 1:50 or 1:200 before added to the microtiter plate wells. Incubation temperature was set to 30 ºC and the microtiter plates were incubated for 5-6 hours for indicator strains diluted 1:50 and ON for indicator strains diluted 1:200. The indicator strains used when assaying the mutant PlnE peptides were *Lactobacillus viridescens* NCDO 1655 and *Lactobacillus curvatus* 89 LMGT 2355, both grown in the presence of MRS medium.

The minimum inhibitory concentration value (MIC value) was defined as the amount of wild type or peptide variants of PlnE that inhibited the growth of the indicator strain by 50%, under the above mentioned conditions. In other words, the wild type PlnE peptide (or the mutant PlnE peptide) is the one which is being quantitated in terms of MIC values. The concentration of wild type PlnF was applied to the wells in a molar excess compared to the concentration of wild type or mutant PlnE peptides.

4. Results

4.1 Construction of the pPlnE100 Plasmid

The expression plasmid, pPlnE100, was constructed for production and expression of wild type and peptide variants of PlnE without its complementary peptide, PlnF, for easier isolation and purification from a culture medium. The sakacin P leader sequence was fused to the *plnE*-gene to complement the pSAK20 plasmid necessary for transcriptional regulation and secretion of the bacteriocin (*98*). The *plnI*-gene encoding the plantaricin EF immunity protein was also included in the pPlnE100 plasmid downstream of the *plnE*-gene to ensure protection of the producer strain. Both the *plnE*-gene fused to the sakacin P leader sequence and the *plnI*-gene were placed under control of the sakacin A promoter. Plasmid charts of pPlnE100 and pSAK20 are shown in the Appendix, section 6.6.

4.1.1 Synthesis of Insert by PCR

The megaprimer method (section 3.3.2) was used to fuse the sakacin A promoter and sakacin P leader sequence to the *plnE*-gene (Megaprimer 1, 334 bp; Fig 4.1 A), resulting in a product of 434 bp, termed Megaprimer 2 (Fig 4.1 B). The next step was to amplify the *plnI*-gene from the genomic DNA of *L. plantarum* C11 and at the same time introduce a restriction site for the restriction enzyme *Cla*I downstream of the *plnI*-gene (Fig 4.1 C). The size of the product was expected to be 970 bp long. This product was then spliced together with Megaprimer 2 by PCR SOEing (section 3.3.3) and amplified in the final PCR reaction (Fig 4.1 D), creating the final PCR product with a length of 1374 bp. All the PCR products were purified using a GFX kit (section 3.2.3) after gel electrophoresis (section 3.4). The correct sequences were verified by DNA sequencing. The sequencing primers are listed in the Appendix, section 6.4.1.

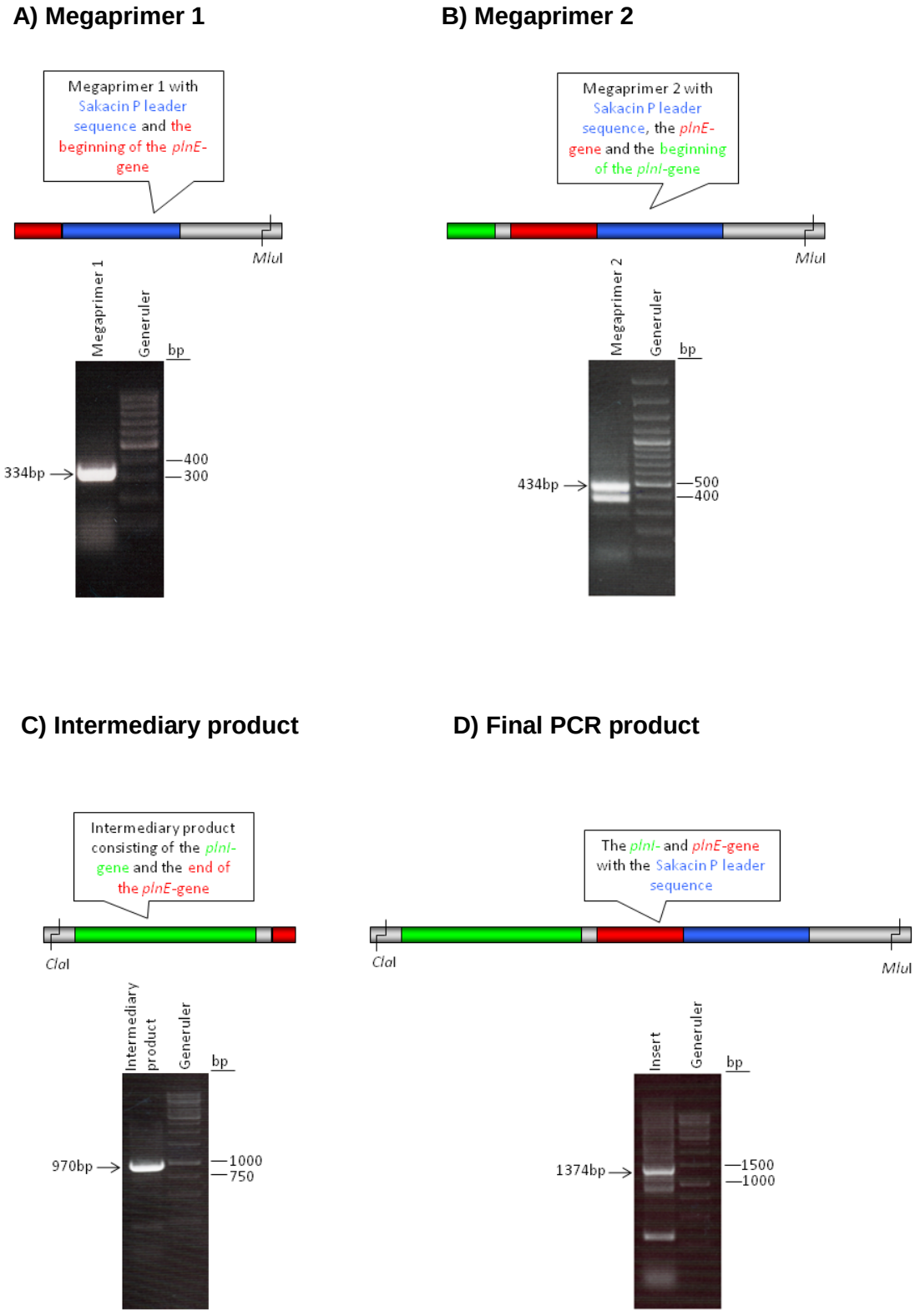


Fig 4.1 Verification of the four PCR products by agarose gel electrophoresis. A) The PCR product of Megaprimer 1. **B)** The PCR product of Megaprimer 2. **C)** An intermediary PCR product which overlaps with Megaprimer 2. **D)** The final PCR product (the insert).

4.1.2 Ligation of Insert into Vectors

It has been shown that ligation reactions involving the pLPV111 plasmid together with an insert containing the same sequence as in this thesis with the exception of exchanging the *plnE*-gene with its complementary two-peptide bacteriocin gene, *plnF*, is unsuccessful in that the plasmid will re-ligate rather than integrate the insert (*106*). Both the plasmid and insert were cut with the same restriction enzymes (*Mlu*I and *Cla*I) as was done in this study. According to the study, the linearized plasmid was then dephosphorylated in order to inhibit re-ligation, but with no marked improvement (*106*). The unsuccessful ligation reactions may be due to poor digestion of the *Cla*I-site in the insert, since this restriction site is approximately 10 bp to the 3'-end of insert (Fig 4.2).

```
5'-acactttatgcttccggctcgtatgttgtgtggaattgtgagcggataacaatttc
acacaggaaacagctatgaccatcgttacgccaagctatttaggtgacactatagaata
ctcaagctatgcatccaacgcgtgaattcccctgtttaggaatgatttctgtaggcttc
aagaagttatgccacgtgatcaaagaaatcttgttatacttcactcgtacaaaaaataa
taacagaggagattcttagttatggaaaagtttattgaattatctttaaaagaagtaac
agcaattacaggtggatttaatcggggcggttataactttggtaaaagtgttcgacatg
ttgttgatgcaattggttcagttgcaggcattcgtggtattttgaaaagtattcgttaa
gctcagccttttagtttatatgggggaattttaactatgccattaataaaagtaata
atcacagctatttactacctaatagtgctctttttaatgaatcctttgactgatattat
gggaataaaagacggacccttcaattcatattaactgaatctattatattaatcgcga
taatcattctgaataggcgatatgttaagcaacctatccactggctacccgttaatatc
atgtcaatattaaagaagaatagtctgcctttgagcctagcaataatatttctacttat
tttctttcgaaaccacatgtatcaatttttaatctcattattactttcgttgattgtag
cgataactgaagaatacgcttttcgtggcatgatattcagaacgcttttagcactaaac
cttaaaaaatttgccacattgcaggccaccatcgcttcgatgatgactgcttcgcttat
ttttgcggctatgcatttggtcaacctttatcacagcccgtatggtcagtattgtgtc
aggttctctatgttattgggttaggtatttacttgcagcaatctatttaaaaaccgga
agcttactggctgccattagtgtccattggttgatagatttttctagtttctattctca
aggtattgacccaactcaatcacccattaacgggccaatggaggctcttctaaaggggc
tctttctaaatattttattcattggcattgctacgtttatcctatcctctaagcattgg
aagttattgagtatcctaaatattgaagataaaatagacgagtaatcatccattctgaa
ttcctataaattattgtttgcagcactacaatttcaattatctctgaagttttgggtta
cgctaagagctagtcatatcgctaactaaatttcacgtcttaacgctatgctaatatac
tttacgatcgattactgtgc-3'
```

Fig 4.2 The nucleotide sequence of the final PCR product (insert). The sakacin P leader sequence is outlined in blue, the *plnE*-gene is outlined in red and the *plnI*-gene is outlined in green. The restriction sites for *Mlu*I and *Cla*I are outlined and underlined in purple and orange, respectively.

Generally, a cleavage site should not be located too close to the end of the linear DNA in order to obtain efficient cleavage (*97*). Sub-cloning into the pGEM®-T Easy Vector,

using the principle of TA-cloning, was shown to circumvent this problem (*106*). For that reason, sub-cloning into the pGEM®-T Easy Vector was also used in this study.

The insert was treated with *Taq* DNA Polymerase (Fermentas) to create 3' terminal deoxyadenosine overhangs (section 3.3.5) prior to ligation (section 3.2.6) into the pGEM®-T Easy Vector. The ligation reaction was transformed into DH5α cells (section 3.1.2) and grown on LB agar plates in the presence of ampicillin for selection (section 3.1.5). Plasmids from isolated clones (section 3.2.2) were screened for insert by double restriction digestion with *Mlu*I and *Cla*I and verified by DNA sequence analysis (section 3.2.7). The restriction digestion produced the correct size of insert (results not shown). In two separate solutions, the pGEM®-T Easy Vector containing the correct insert and the pLT100β plasmid (a pLPV111 derivative) were treated with a double restriction digestion with *Mlu*I and *Cla*I and screened by gel electrophoresis (results not shown). The band corresponding to the correct size of insert and the band corresponding to the linear pLPV111 plasmid were excised from the gel and purified with a GFX kit. The insert was then ligated into the pLPV111 plasmid using 1:1, 3:1 and 5:1 insert:plasmid molar ratios and transformed into DH5α cells. The cells were grown on LB agar plates in the presence of erythromycin. Purified plasmids with insert were verified by DNA sequencing. The insert turned out to be correctly ligated with the exception of a silent mutation in the codon for glutamic acid, position 218 (E218), in the *plnI*-gene.

4.2 Construction of the Peptide Variants

Both CD and NMR analysis have shown that plantaricin EF induces helical structuring when exposed to membrane-like entities such as micelles and negatively charged liposomes (*3, 58*). As described in section 1.5.4, all two-peptide bacteriocins characterized to date contain GxxxG motifs (*27*). To investigate the importance of the GxxxG motifs in plantaricin EF, point mutations were introduced by site-directed mutagenesis in the two GxxxG motifs of the PlnE peptide, G_5xxxG_9 and $G_{20}xxxG_{24}$. The glycine residues were substituted one at a time in both motifs with alanine, serine, lysine, glutamine, leucine and isoleucine residues. Also, in order to see how the structure of the membrane-bound plantaricin EF was affected by the tyrosine residue in PlnE (Y6; Fig 4.3), substitutions with the amino acids tryptophan, phenylalanine,

leucine and arginine were performed. Fig 4.3 demonstrates the nucleotide and amino acid sequences of the PlnE peptide fused to the sakacin P leader sequence (upper row). The G_5xxxG_9 and the $G_{20}xxxG_{24}$ motifs are marked with grey background and the mutation sites are marked in green. All the mutated peptides were assigned a name based on the original amino acid, position and the substituted amino acid. If, for instance, a glycine residue at position 5 is exchanged with an alanine residue, the mutated peptide is termed ‘G5A’.

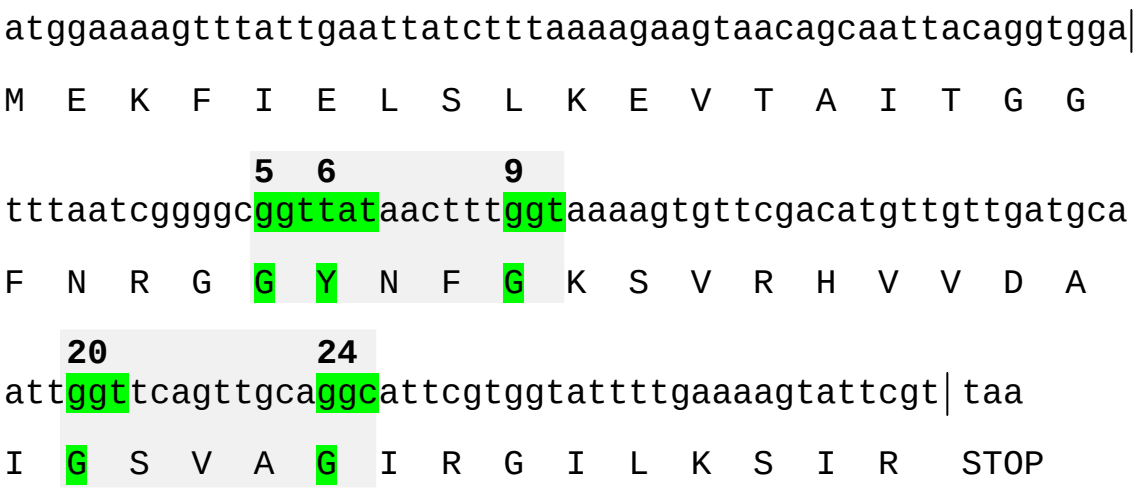


Fig 4.3 Nucleotide and amino acid sequences of PlnE and the sakacin P leader sequence. The sakacin P leader sequence is displayed in the upper row followed by the PlnE peptide. The two GxxxG motifs are marked with grey background. The tyrosine residue is at position 6 and all mutation sites are marked in green.

A total of 28 mutants were constructed by *in vitro* site-directed mutagenesis (section 3.3.4). The PCR product was treated with *Dpn*I to digest the methylated parental plasmid DNA (section 3.2.5) and transformed into DH5α cells and subsequently grown on LB plates containing erythromycin (section 3.1.5). Purified plasmids were sequenced for verification and 27 out of 28 mutations were confirmed. The only mutation not possible to verify was the substitution of Y6 with a tryptophan residue (i.e. the Y6W mutation). Table 4.1 summarizes the constructions, transformations and verification by sequencing of the mutated pPlnE100 plasmids. Two sequencing primers specific for the pLPV111 plasmid were used (Appendix, section 6.4.1), pLPV111sekF and pLPV111sekR (both from Eurogentec S.A.). They anneal to each strand of the plasmid upstream the restriction sites for *Cla*I and *Mlu*I, respectively. All the mutagenic primers are listed in the Appendix, section 6.4.2.

Table 4.1 Construction, transformation and verification by DNA sequencing of the mutated pPlnE100 plasmids:

Mutation	PCR product of mutated pPlnE100 observed on agarose gel	Mutated pPlnE100 transformed into *E. coli* DH5α as observed on agarose gel	Mutated pPlnE100 verified by sequencing
G_5xxxG_9 Motif			
G5A	+	+	+
G5S	+	+	+
G5K	+	+	+
G5Q	+	+	+
G5L	+	+	+
G5I	+	+	+
G9A	+	+	+
G9S	+	+	+
G9K	+	+	+
G9Q	+	+	+
G9L	+	+	+
G9I	+	+	+
$G_{20}xxxG_{24}$ Motif			
G20A	+	+	+
G20S	+	+	+
G20K	+	+	+
G20Q	+	+	+
G20L	+	+	+
G20I	+	+	+
G24A	+	+	+
G24S	+	+	+
G24K	+	+	+
G24Q	+	+	+
G24L	+	+	+
G24I	+	+	+
Tyrosine Residue			
Y6L	+	+	+
Y6R	+	+	+
Y6F	+	+	+
Y6W	+	+	-*
Total	28	28	27

'+'/'-' Designates that the work has been accomplished/not accomplished.

* Not possible to verify the mutation Y6W by DNA sequencing due to large noise in peak reading.

4.3 Production of the Peptide Variants

The peptides were produced by the use of the heterologous expression system *L. sakei* Lb790/pSAK20/pPlnE100 (section 3.5.1). When all the purified pPlnE100 plasmids containing the desired mutated *plnE*-gene had been verified by sequencing, they were transformed into competent *L. sakei* Lb790/pSAK20 cells by electroporation (section 3.1.4). Successfully transformed cells were grown on MRS plates containing the appropriate antibiotics for selection of pPlnE100 and pSAK20 (section 3.1.5). Agarose gel electrophoresis accompanied by DNA sequence analysis with purified pPlnE100 plasmids from *L. sakei* Lb790/pSAK20 cells verified that the cells had been transfected with the correct plasmid (results not shown; see Table 4.3, section 4.4.4 for an overview of all constructed mutants).

4.4 Purification and Analysis of the Peptide Variants

The purification steps and all downstream applications in this thesis were performed with the wild type PlnE and PlnF peptides as well as peptides with mutations in the G_5xxxG_9 motif with the exception of the mutation G9I, which was only purified.

4.4.1 The Two-Step Purification Procedure

The first of two purification steps carried out on wild type and mutant PlnE peptides was performed by the use of cation-exchange chromatography (section 3.5.3). All eluted peptides were confirmed present in activity measurements upon complementation with wild type PlnF. The peptides were further purified by reverse-phase chromatography (section 3.5.4) where the UV absorbance was recorded at 214 nm and 280 nm. A representative collection of chromatograms is shown in Figs 4.4 to 4.8. All the peptides eluted between 38.5% and 42% 2-propanol. Correctly mutated peptides were verified by MS analysis.

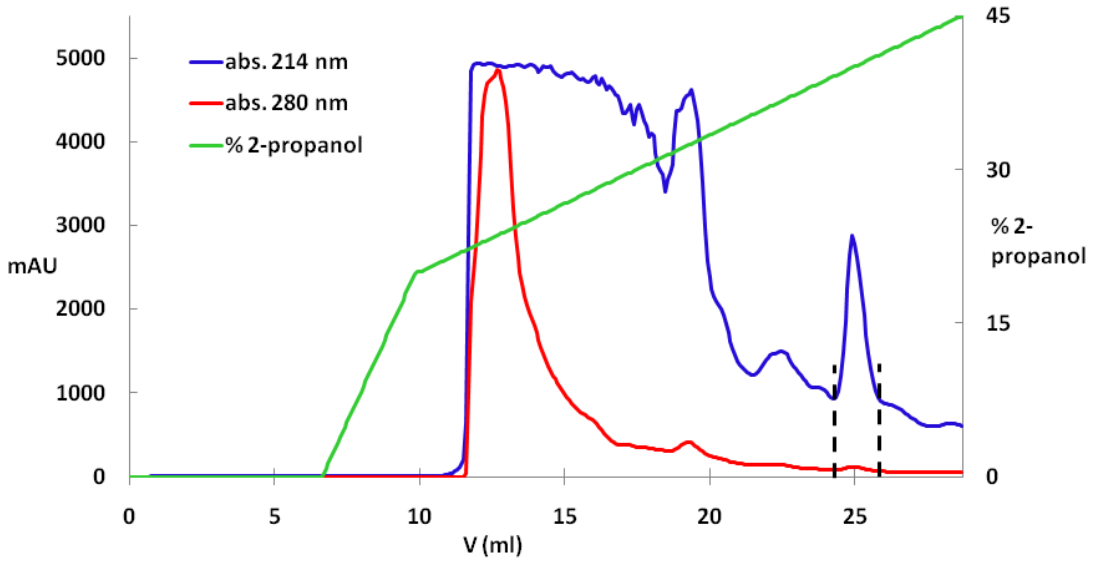


Fig 4.4 Reverse-phase chromatogram of the PlnE peptide. The PlnE peptide eluted at approximately 39% 2-propanol. The correct peak (i.e. fraction with antimicrobial activity) is indicated with vertical, stapled lines. The absorbance-scale (i.e. milli absorbance units (mAU)) on the vertical axis represents both 214 nm and 280 nm. The horizontal scale represents the elution volume (V) in ml. Confirmation of true identity was verified by MS analysis and activity measurements.

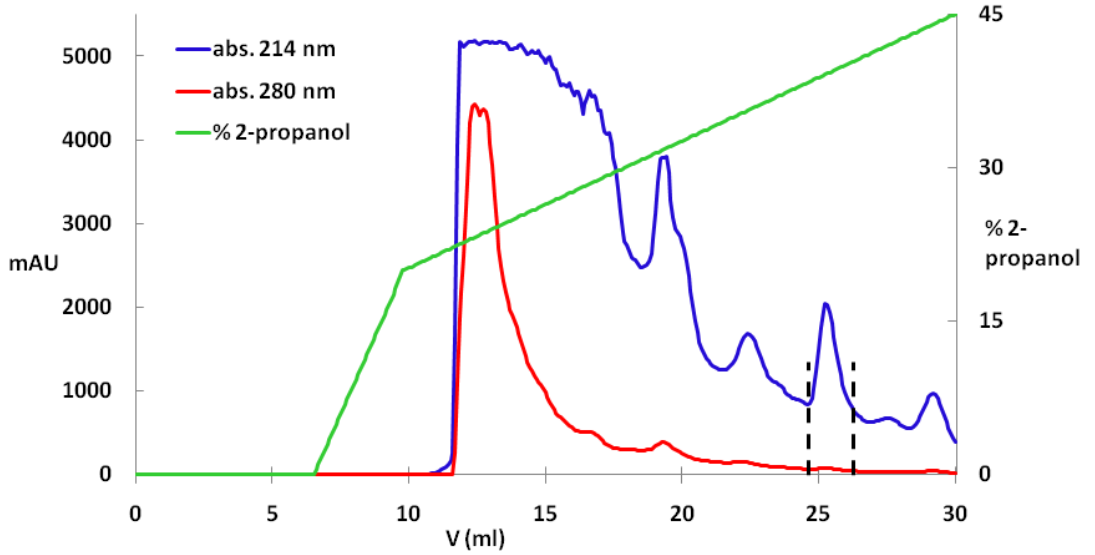


Fig 4.5 Reverse-phase chromatogram of the G5A peptide. The G5A peptide eluted at approximately 39% 2-propanol. See Fig 4.4 for description of chromatogram.

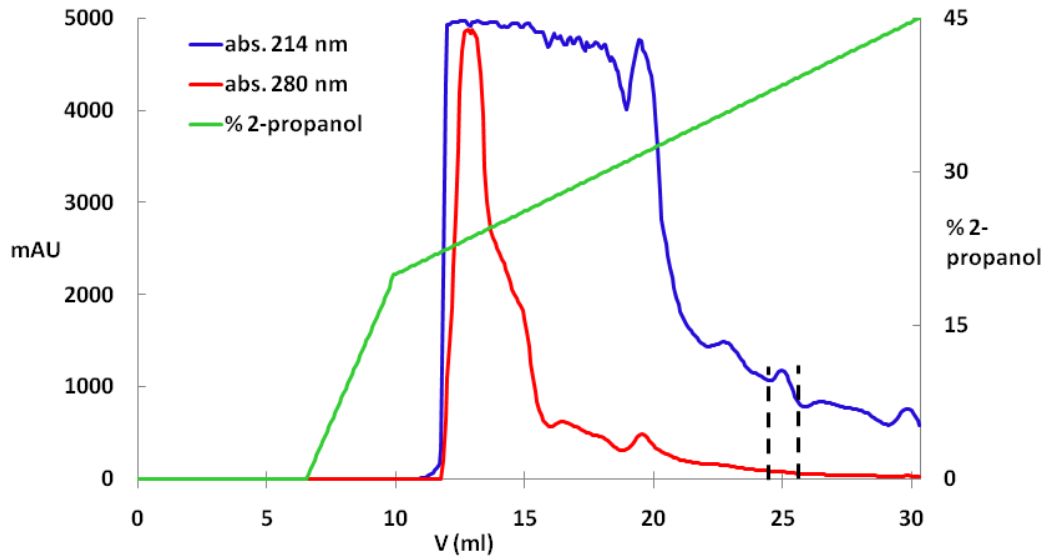


Fig 4.6 Reverse-phase chromatogram of the G5K peptide. The G5K peptide eluted at approximately 38.5% 2-propanol. See Fig 4.4 for description of chromatogram.

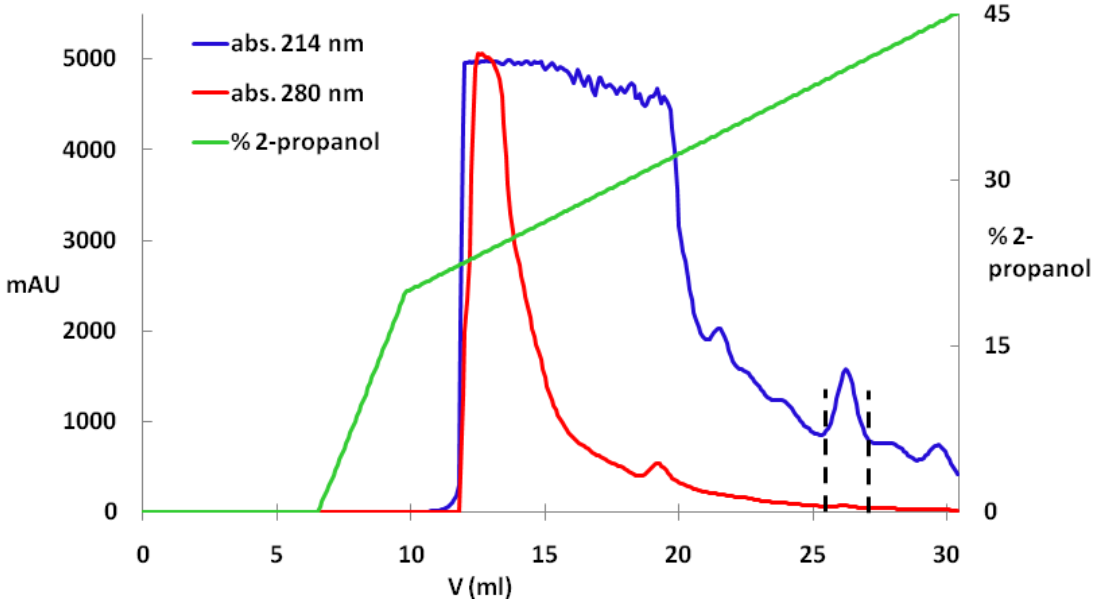


Fig 4.7 Reverse-phase chromatogram of the G9A peptide. The G9A peptide eluted at approximately 40% 2-propanol. See Fig 4.4 for description of chromatogram.

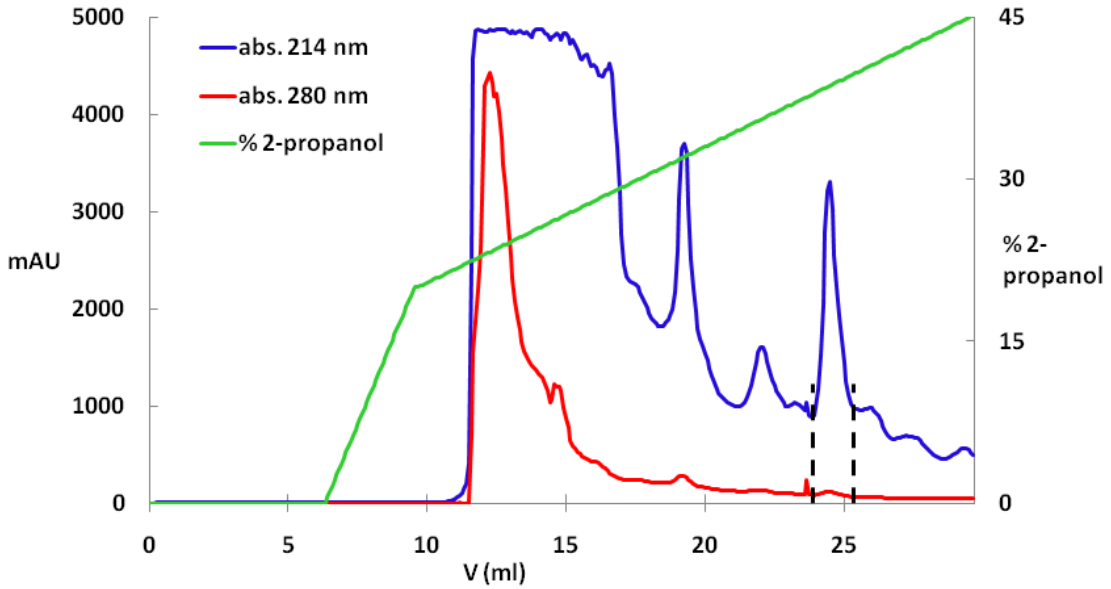


Fig 4.8 Reverse-phase chromatogram of the G9Q peptide. The G9Q peptide eluted at approximately 39% 2-propanol. See Fig 4.4 for description of chromatogram.

4.4.2 Mass Determination

MALDI-TOF MS (section 3.5.6) was used to analyze if the samples contained the desired wild type or mutant PlnE peptides. The measured molecular masses of all the collected compounds are shown in Table 4.2 and were in good agreement with the theoretical masses.

Table 4.2 The theoretical and detected masses of the wild type and mutant PlnE peptides:

Peptide	Theoretical mass (Da)	Detected mass (Da)
PlnE	3545	3544
G5A	3559	3558
G5S	3575	3574
G5K	3616	3615
G5Q	3616	3615
G5L	3601	3600
G5I	3601	3600
G9A	3559	3558
G9S	3575	3574
G9K	3616	3615
G9Q	3616	3615
G9L	3601	3600

4.4.3 Degree of Purity

The purification analysis of the wild type and peptide variants of PlnE was performed by analytical reverse-phase chromatography using the SMART system (Amersham Biosciences) as described in section 3.5.5. A collection of chromatograms is shown in Figs 4.9 to 4.12. The peptides eluted between 30% and 36% (v/v) 2-propanol/0.1% (v/v) TFA and the fractions were subsequently analyzed by MS for verification of correct peptide. Usually, a volume of 200 µl sample was diluted with 800 µl dH_2O/0.1% (v/v) TFA to decrease the concentration of 2-propanol before applying to the column. Judged by the chromatograms, most of the peptides were estimated to have a purity of over 70% except for the mutant G9A which was estimated to have a purity of approximately 50% because of a second peak (as well as two other small peaks in front) eluting close to the putative G9A peptide peak with no baseline separation (Fig 4.12). The mass spectra obtained from these fractions of the G9A solution showed, however, no other components in the sample other than the G9A peptide and hence the purity is probably higher than initially assumed.

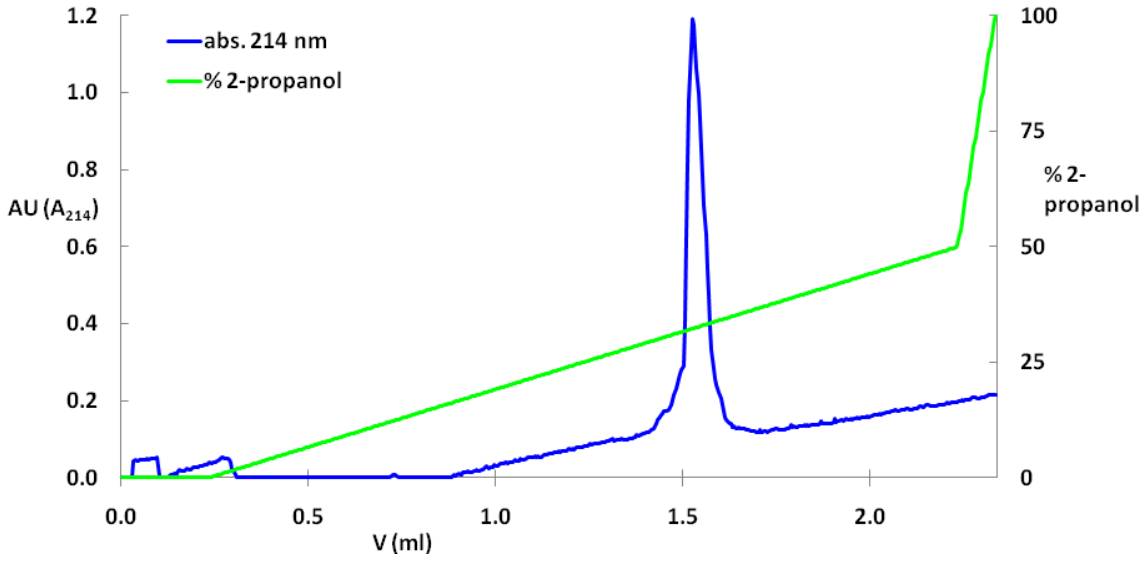


Fig 4.9 Analytical reverse-phase chromatogram of the PlnE peptide using the SMART system. The PlnE peptide eluted at approximately 30% 2-propanol. The absorbance-scale (i.e. absorbance units (AU)) represents the absorbance at 214 nm and the horizontal scale represents the elution volume (V) in ml.

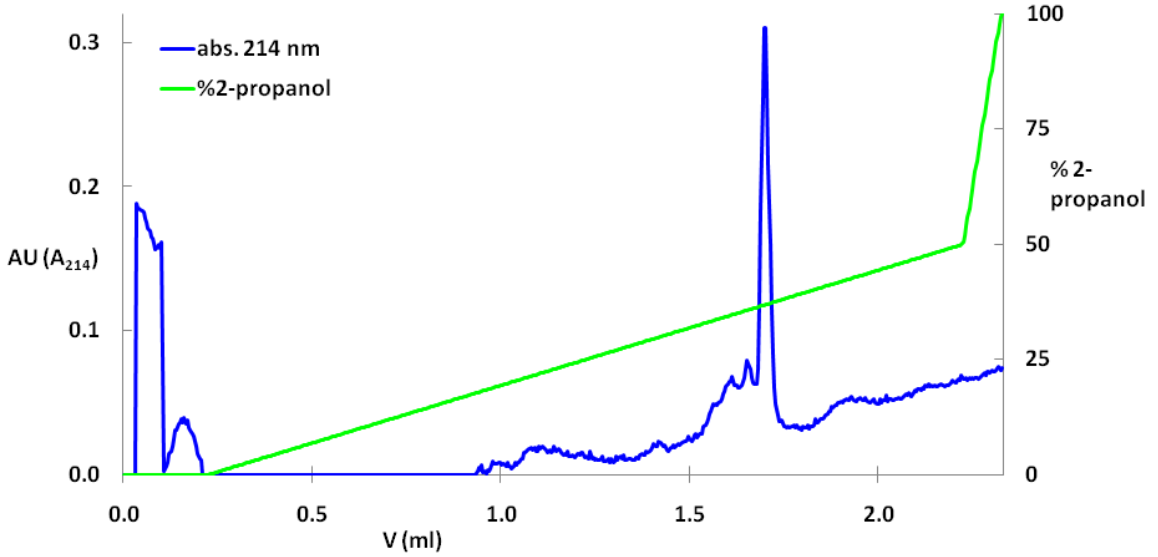


Fig 4.10 Analytical reverse-phase chromatogram of the G5A peptide using the SMART system. The G5A peptide eluted at approximately 35% 2-propanol. See Fig 4.9 for description of chromatogram.

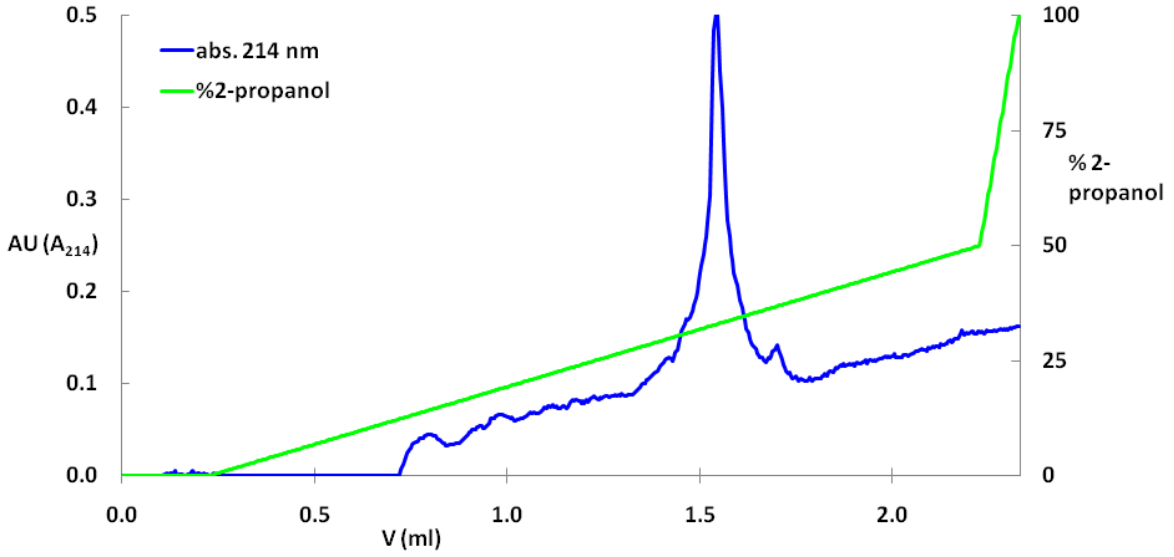


Fig 4.11 Analytical reverse-phase chromatogram of the G5K peptide using the SMART system. The G5K peptide eluted at approximately 30% 2-propanol. See Fig 4.9 for description of chromatogram.

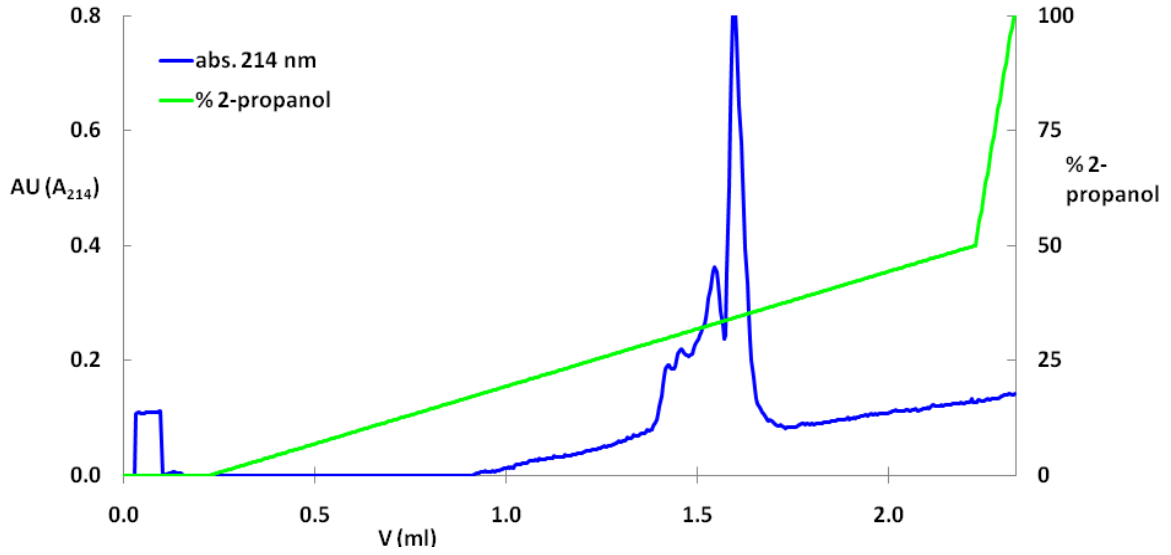


Fig 4.12 Analytical reverse-phase chromatogram of the G9A peptide using the SMART system. Several peaks were obtained. The G9A peptide eluted at approximately 32.5% 2-propanol (the peak with most absorbance), whereas the second largest peak eluted at approximately 31.5% 2-propanol. See Fig 4.9 for description of chromatogram.

4.4.4 Summary of Constructs and Purified Peptide Variants of PlnE

The 24 mutated pPlnE100 plasmids constructed based on the two GxxxG motifs in PlnE were transformed into *L. sakei* Lb790/pSAK20 cells and verified by DNA sequencing. Peptide expression of the 12 variants based on the G_5xxxG_9 motif was verified by MS analysis as well as confirmation of activity in bacteriocin activity assays, except for the mutant G9I which was not applied in activity assays. With the exception of the mutation Y6W, all mutated pPlnE100 plasmids constructed based on the tyrosine residue were transformed into *E. coli* DH5α cells and verified by DNA sequencing. Table 4.3 summarizes confirmed transformations, peptide expression, and verification by MS analysis and activity assays of all the PlnE variants that were constructed.

Table 4.3 An overview of the pathway from transformation into cells to verification of peptide expression by MS analysis and activity assays:

Mutation	Verified transformation into *E. coli* DH5α	Transformed into *L. sakei* Lb790/ pSAK20	Verified by sequence	Purified peptide	Verified by MS	Confirmed activity with assays	Analyzed degree of purity
G_5xxxG_9 Motif							
G5A	+	+	+	+	+	+	+
G5S	+	+	+	+	+	+	+
G5K	+	+	+	+	+	+	+
G5Q	+	+	+	+	+	+	+
G5L	+	+	+	+	+	+	+
G5I	+	+	+	+	+	+	+
G9A	+	+	+	+	+	+	+
G9S	+	+	+	+	+	+	+
G9K	+	+	+	+	+	+	+
G9Q	+	+	+	+	+	+	+
G9L	+	+	+	+	+	+	+
G9I	+	+	+	+	+	-[a)]	-
G_{20}xxxG_{24} Motif							
G20A	+	+	+	-[b)]	-	-	-
G20S	+	+	+	-	-	-	-
G20K	+	+	+	-	-	-	-
G20Q	+	+	+	-	-	-	-
G20L	+	+	+	-	-	-	-
G20I	+	+	+	-	-	-	-
G24A	+	+	+	-	-	-	-
G24S	+	+	+	-	-	-	-
G24K	+	+	+	-	-	-	-
G24Q	+	+	+	-	-	-	-
G24L	+	+	+	-	-	-	-
G24I	+	+	+	-	-	-	-

Table 4.3 continued:

Mutation	Verified transformation into *E. coli* DH5α	Plasmids transformed into *L. sakei* Lb790/ pSAK20	Verified by sequence	Purified peptide	Verified by MS	Confirmed activity with assays	Analyzed degree of purity
Tyrosine Residue							
Y6L	+	-[c]	-	-	-	-	-
Y6R	+	-	-	-	-	-	-
Y6F	+	-	-	-	-	-	-
Y6W	-	-	-	-	-	-	-
Total	27	24	24	12	11	11	11

'+'/'-' Designates that the work has been accomplished/not accomplished.

[a] The mutant G9I was not included in activity assays.

[b] The mutated pPlnE100 plasmids constructed based on the $G_{20}xxxG_{24}$ motif were only confirmed properly transformed into *L. sakei* Lb790/pSAK20.

[c] The mutated pPlnE00 plasmids constructed based on the tyrosine residue (mutations Y6L, Y6R and Y6F, not Y6W) were only confirmed properly transformed into *E. coli* DH5α (as verified by DNA sequencing).

4.4.5 Estimated Peptide Concentrations

As described in section 3.5.7, the OD at 214 nm of the wild type and mutant peptides of PlnE were estimated based on their peak areas from chromatograms obtained by reverse-phase chromatography using the ÄKTA system. This was done because almost none of the mutant peptides had high enough concentrations to be measured at an absorbance of 280 nm. The extinction coefficient of PlnE at 214 nm was calculated to be approximately 50000 $M^{-1}cm^{-1}$ and it was assumed that this value would also hold true for the mutant peptides. Consequently, the concentration of all peptides was calculated by the use of this extinction coefficient and their OD at 214 nm. The results are listed in Table 4.4.

Table 4.4 Measured absorbance at 214 nm for the wild type and peptide variants of PlnE and their calculated concentrations as well as the amount of peptide isolated per L cell culture:

Peptide	A_{214} [a)]	Concentration (µM)	Amount peptide isolated per L cell culture (µg)
PlnE	0.6	12	190
G5A	0.3	6	90
G5S	0.1	2	15
G5K	0.05	1	10
G5Q	0.5	10	100
G5L	0.07	1	13
G5I	0.2	4	40
G9A	0.2	4	40
G9S	0.3	6	60
G9K	0.05	1	8
G9Q	0.2	4	130
G9L	0.4	8	110

[a)] The UV absorbance of each peptide at 214 nm based on the peak area from the respective chromatograms obtained using the ÄKTA system.

4.5 Bacteriocin Activity Assays

The bacteriocin activity assays were performed as described in section 3.5.8. A purified wild type PlnF peptide (known to be devoid of contaminants) obtained from a previous study by Fimland *et al.* (*61*) was used in all assays performed on the mutant PlnE peptides.

4.5.1 Finding Suitable Indicator Strains

Several different indicator strains were assayed against wild type PlnE and PlnF to find a suitable indicator strain with high sensitivity toward the bacteriocin. The results are

listed in Table 4.5 as well as the incubation conditions for all the indicator strains tested. Three independent parallels were used. The two most sensitive indicator strains, *Lactobacillus viridescens* NCDO 1655 and *Lactobacillus curvatus* 89 LMGT 2355, were chosen to evaluate activity of the different mutant PlnE peptides.

Table 4.5 Possible indicator strains tested against PlnE+PlnF:

Organism	Strain	Mean MIC value[a)] (nM)	Incubation conditions
Carnobacterium piscicola	LMGT 2332	430	MRS medium, ON
Lactobacillus corynifornis ssp. *Torquens*	NCDO 2740	30	MRS medium, 6 hours
Lactobacillus curvatus 89	LMGT 2355	1	MRS medium, 6 hours
Lactobacillus plantarum 965	LMGT 2003	5	MRS medium, 6 hours
Lactobacillus sakei	NCDO 2714	20	MRS medium, ON
Lactobacillus viridescens	NCDO 1655	2	MRS medium, 6 hours
Lactococcus	LMGT 2077	160	GM17[b)] medium, ON
Lactococcus lactis	MG 1363	180	GM17[b)] medium, 6 hours
Lactococcus lactis	IL 1403	170	GM17[b)] medium, 6 hours
Pediococcus acidilactici	LMGT 2351	30	MRS medium, 6 hours
Pediococcus acidilactici	NCDO 521	30	MRS medium, ON
Pediococcus acidilactici	NCDO 1859	400	MRS medium, 6 hours
Pediococcus pentosaceus	NCDO 990	50	MRS medium, ON
Pediococcus pentosaceus FBB 63 B	LMGT 2722	400	MRS medium, 6 hours

[a)] The MIC value is defined as the concentration of the wild type PlnE that inhibits the growth of the indicator strain by 50% when PlnE is present together with a molar excess of PlnF.
[b)] M17 broth supplemented with 0.4% (v/v) glucose and 0.1% (v/v) Tween 80.

4.5.2 Bacteriocin Activity of Peptide Variants of PlnE Assayed Against *Lactobacillus viridescens* NCDO 1655

The indicator strain *Lactobacillus viridescens* NCDO 1655 was assayed against the mutant PlnE peptides complemented with wild type PlnF. The wild type PlnE+PlnF combination was also assayed for comparison. The results are listed in Table 4.6 and the MIC values represented are obtained from three independent parallels.

Assaying the combinations G9K+PlnF and G9S+PlnF resulted in a 30 to 60 fold reduction in activity, whereas the G9L+PlnF combination caused a 150 fold reduction in activity compared to the wild type combination. The combination G9Q+PlnF was highly detrimental as the activity was reduced by over 300 times. Reasonably well tolerated combinations (3 to 13 fold reduction in activity) were also observed: G9A+PlnF, G5K+PlnF, G5L+PlnF and G5I+PlnF. Interestingly, the G5A+PlnF combination resulted in an approximately 2 fold increase in activity compared to the wild type combination. G5S+PlnF resulted in a similar activity as the wild type combination.

When comparing equivalent amino acid changes in the G5 position versus the G9 position in the G_5xxxG_9 motif, changes in the G9 position seemed to have the most detrimental effect on activity toward the indicator strain when combined with wild type PlnF.

Table 4.6 MIC values of mutant PlnE peptides complemented with PlnF assayed against *Lactobacillus viridescens* NCDO 1655:

Peptide[a)]	**MIC value (nM)**[b)]	$\frac{\text{MIC(mut)}}{\text{MIC(wt)}}$[c)]
PlnE+PlnF	0.1/0.2/0.5	1
G5A+PlnF	0.1/0.1/0.2	0.5
G5S+PlnF	0.2/0.1/0.3	0.8
G5K+PlnF	2/3/3	10
G5Q+PlnF	20/40/30	100
G5L+PlnF	0.7/1/1	3
G5I+PlnF	1/2/2	6
G9A+PlnF	3/3/4	13
G9S+PlnF	16/14/18	60
G9K+PlnF	8/6/7	30
G9Q+PlnF	100/80/110	360
G9L+PlnF	40/40/40	150

[a)] A purified PlnF peptide from a previous study by Fimland *et al.* (*61*) was used in all mutant peptide assays.

[b)] The MIC value is defined as the concentration of the wild type or peptide variants of PlnE that inhibits the growth of the indicator strain by 50% when one of these variants or wild type PlnE are present together with a molar excess of PlnF. The three values in each row represent the MIC value from three independent parallels.

[c)] The mean MIC value of the mutant peptide divided by the mean MIC value of the wild type PlnE peptide. This is a relative value which indicates the difference in activity of the mutant peptide compared to the wild type peptide, normally as an 'X' fold reduction in activity where 'X' is the relative value.

4.5.3 Bacteriocin Activity of Peptide Variants of PlnE Assayed Against *Lactobacillus curvatus* 89 LMGT 2355

The indicator strain *Lactobacillus curvatus* 89 LMGT 2355 was also assayed against the mutant peptides complemented with wild type PlnF. Only two independent parallels were run for each combination. Although three independent parallels is normally the minimum required for a good quantitation of results, the two independent parallels of each mutant peptide confirmed a quite similar value. As an example, the peptide combination G5L+PlnF had a MIC value of 0.1 the first day and a value of 0.3 the next day. An exception was the PlnE+PlnF combination which had a MIC value of 0.02 the first day and a value of 0.2 the next day (a 10 times increase in MIC value). The results are listed in Table 4.7.

The G9L+PlnF and the G9Q+PlnF combinations were the most detrimental, resulting in 180 to 600 fold reduction in activity, respectively. The only G5 mutant that was not well tolerated was G5Q which, when complemented with PlnF, resulted in a 120 fold reduction in activity. Other G5 mutants (G5S, G5K, G5L and G5I), as well as the G9A mutant, were reasonably well tolerated when complemented with PlnF, causing only 2 to 9 fold reduction in activity. Regarding the rest of the G9 mutant combinations, G9K+PlnF and G9S+PlnF resulted in a 40 to 70 fold reduction in activity, respectively. Although assayed only once with this indicator strain, the G5A+PlnF combination showed a 4 fold increase in activity compared to PlnE+PlnF, approximately equal to the results observed for the same combination assayed against the indicator strain *Lactobacillus viridescens* NCDO 1655 (Table 4.6).

Compared to the other indicator strain (section 4.5.2), the observations indicates again that the amino acid substitutions in the G9 position results in a higher loss of activity compared to the equivalent changes in the G5 position.

Table 4.7 MIC values of mutant peptides complemented with PlnF assayed against *Lactobacillus curvatus* 89 LMGT 2355:

Peptide[a)]	**MIC value (nM)**[b)]	**$\frac{\text{MIC(mut)}}{\text{MIC(wt)}}$**[c)]
PlnE+PlnF	0.02/0.2	1
G5A+PlnF	<0.01/0.04[d)]	0.4
G5S+PlnF	0.2/0.2	2
G5K+PlnF	1/1	9
G5Q+PlnF	14/13	120
G5L+PlnF	0.1/0.3	2
G5I+PlnF	0.1/0.5	3
G9A+PlnF	1/1	9
G9S+PlnF	7/8	70
G9K+PlnF	4/5	40
G9Q+PlnF	60/70	600
G9L+PlnF	18/22	180

[a)] A purified PlnF peptide from a previous study by Fimland *et al.* (*61*) was used in all mutant peptide assays.

[b)] The MIC value is defined as the concentration of the wild type or peptide variants of PlnE that inhibits the growth of the indicator strain by 50% when one of these variants or wild type PlnE are present together with a molar excess of PlnF. The two different values indicate the measured MIC values from two independent parallels.

[c)] The mean MIC value of the mutant peptide divided by the mean MIC value of the wild type peptide. This is a relative value which indicates the difference in activity of the mutant peptide compared to the wild type peptide, normally as an 'X' fold reduction in activity where 'X' is the relative value.

[d)] Assaying the G5A peptide resulted in only one measurable MIC value because of high activity.

5. Discussion

5.1 Production and Purification of Wild Type and Peptide Variants of PlnE

A total of 28 mutated pPlnE100 plasmids – 24 involving replacement of the glycine residues in the two GxxxG motifs and 4 involving replacement of the tyrosine residue in position 6 – were constructed by the use of *in vitro* site-directed mutagenesis and were successfully transformed into *E. coli* DH5α cells (Table 4.1). All mutations, except for the Y6W mutation, were verified by DNA sequencing. The Y6W mutation was performed three times by site-directed mutagenesis and the putative constructs were transformed into *E. coli* DH5α cells more than four times. In all the sequencing results obtained with the Y6W construct, large background noise masked all attempts to confirm the correct mutated sequence. Because of time limitations, it was decided to focus the subsequent work on mutations involving the two putative helix-helix interacting GxxxG motifs in PlnE. The 24 mutated variants involving these two motifs were transformed into *L. sakei* Lb790/pSAK20 cells and verified by DNA sequencing (Table 4.3). For the subsequent purification and activity measurements, it was decided (again, because of time limitations) to focus on the 11 peptide variants in which the glycine residues in the G_5xxxG_9 motif were individually substituted. The G9I mutant was not included, since it is expected that the G9L and the G9I mutants will affect the growth inhibition of sensitive cells in a similar manner due to similar/comparable side chain properties. Correctly introduced mutations were verified by DNA sequencing and by MS analysis after purification of the peptides (Table 4.3).

The amount of mutant peptides isolated per 1 L cell culture varied from less than 20 µg (as was the case for the mutants G5S, G5K, G5L and G9K) to 100-130 µg for the mutants G5Q, G9Q and G9L (Table 4.4). The wild type PlnE peptide resulted in a yield of approximately 200 µg. The low yield of some of the mutant peptides can be due to low expression in the host cell and/or poor secretion by the transport machinery. Low yields may also have been due to loss of peptides upon purification. For instance, overloading the cation-exchange column in the first purification step, resulting in peptides simply passing through the column, may have contributed to the low yield.

Some activity was indeed detected in the 'flow-through' fractions, which might be indicative of a saturated column. In a recent study, cation-exchange chromatography of the PlnF peptide resulted in only a 2% yield in the absence of 2-propanol in the elution buffer (*106*), whereas a yield of 40% was obtained in the presence of 20% (v/v) 2-propanol (Oppegård, C., personal communication). There was no further increase in the yield upon increasing the percentage of 2-propanol to 60%. Based on these results, 2-propanol was in this study added to a final concentration of 20% to the elution buffer used upon cation-exchange chromatography. It is not clear why 2-propanol increases the yield of PlnF (and presumably also PlnE). Increased hydrophobicity of eluent might prevent (or disrupt) aggregation of peptides on the column.

The measured peak areas at an absorbance of 214 nm were used for estimating the concentration of purified peptides (section 4.4.5). At this wavelength, however, there are several other substances, such as caramelized sugar from the medium and 2-propanol, that also absorbs light and this may lead to an over-estimation of the amount of peptides. This will in turn lead to an under-estimation of the specific antimicrobial activity. Moreover, some of the mutant peptides resulted in only small peaks, even at 214 nm (e.g. the mutations G5K (Fig 4.6), G5I and G9K), which further complicates quantitation of the purified peptides, which in turn leads to uncertainties in the MIC values.

5.2 Effects of the G_5xxxG_9 Mutations on Bacteriocin Activity

When the wild type PlnE and PlnF combinations (both of which were purified in this study) were initially assayed in search for sensitive indicator strains (Table 4.5), a significantly higher MIC value (approximately 10 times higher) was obtained than the MIC values subsequently obtained for the wild type combination when the mutant peptides were assayed (Tables 4.6 and 4.7). The high MIC value obtained initially (Table 4.5) might be due to an over-estimation of the calculated concentration of PlnF that was used in the initial assay. This may in turn have resulted in the actual concentration of PlnF being too low. If the amount of PlnF applied in the activity assay was less than the amount of PlnE, then some of the PlnE peptides will lack a PlnF-

interacting partner and will thus not be active. Consequently, a higher MIC value of PlnE would be determined in these assays (Table 4.5).

A total of 11 peptide variants with replacements in one of the two glycine residues in the G_5xxxG_9 motif were assayed against two different indicator strains (Tables 4.6 and 4.7). Similar results were obtained with both indicator strains, which increase the credibility of the measurements. However, additional indicator strains should eventually be included in order to ascertain that the results obtained are not strain-dependent, as it has been observed that the effect of mutation on bacteriocin activity may in some cases depend on the indicator strains used (*107-109*). The fact that similar results were obtained with the two indicator strains indicate that they are not atypical, as it is unlikely that both strains show unusual behaviour.

Substituting glycine residues in a helix-helix interacting GxxxG motif with a large amino acid residue is expected to have a negative impact on the bacteriocin activity as there is close contact between the polypeptide backbones of the two α-helices at these positions. Furthermore, these glycine residues are expected to be in the hydrophobic core of the membrane, since a hydrophobic environment will strengthen the interhelical hydrogen bond between the Cα hydrogen atom of a glycine residue and a backbone carbonyl oxygen atom on the opposite α-helix. Two putative helix-helix interacting models have been proposed (a parallel and an anti-parallel) for the two-peptide bacteriocin plantaricin EF upon interaction with target-cell membranes (Fig 5.1) (*85*). In both models, the G_5xxxG_9 motif in PlnE will interact with the flat surface of PlnF formed by residues S16, G19 and P20. A third possible model entails that the $G_{30}xxxG_{34}$ motif in PlnF interacts with the G_5xxxG_9 motif in PlnE in a parallel manner (in an anti-parallel manner, the two negatively charged aspartate residues D17 in PlnE and D22 in PlnF would come close to each other) (*61*).

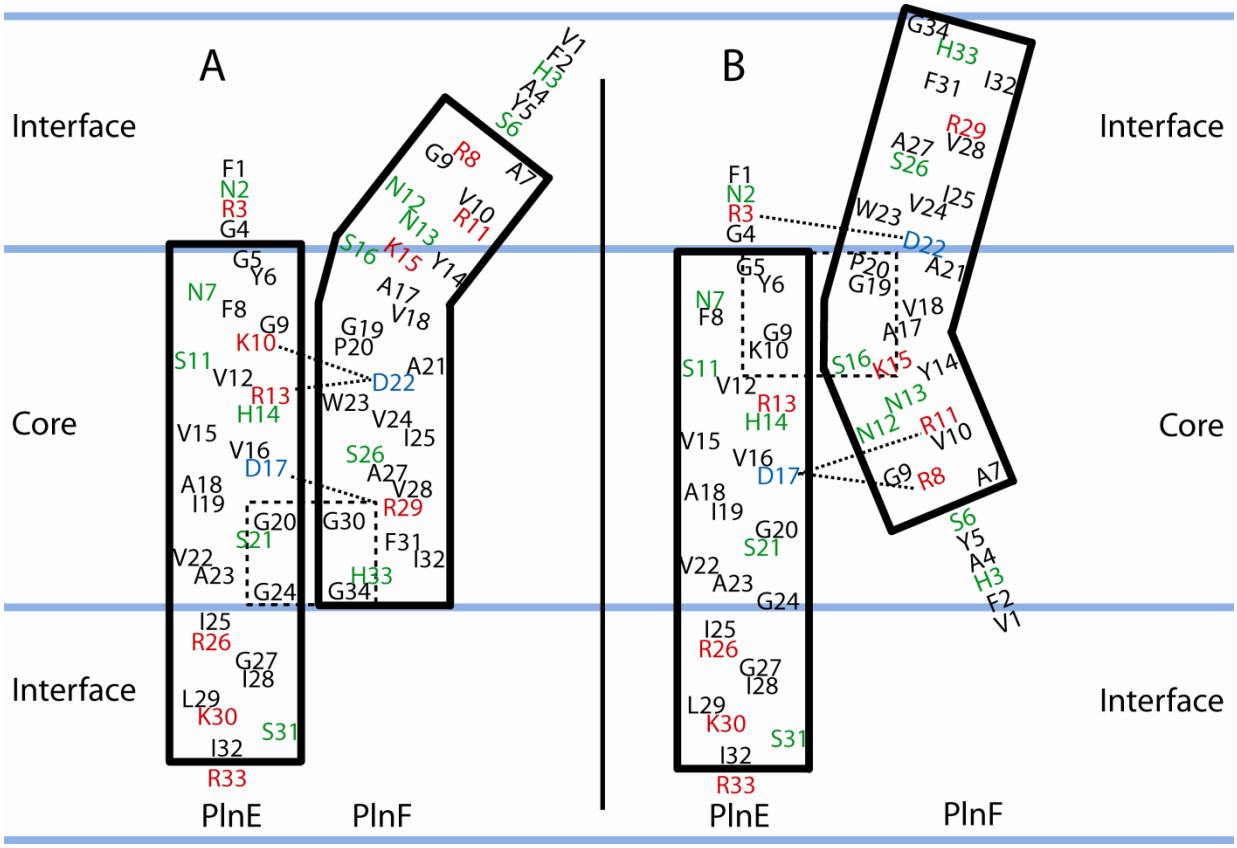


Fig 5.1 Two putative helix-helix interacting models of plantaricin EF. A) PlnE and PlnF interact in a parallel manner where the $G_{20}xxxG_{24}$ motif in PlnE interacts with the $G_{30}xxxG_{34}$ motif in PlnF, enabling the G_5xxxG_9 motif in PlnE to come in close contact with the flat surface of PlnF from residue S16 to P20, thereby creating increased opportunities for charge-charge interactions between the two peptides. **B)** PlnE and PlnF interact in an anti-parallel manner where the G_5xxxG_9 motif in PlnE interacts with the flat surface of PlnF from residue S16 to P20. This will allow for increased charge-charge interactions between the two peptides. Black characters indicate hydrophobic residues, red indicates positively charged hydrophilic residues (K10 in PlnE in B is supposed to be marked as red), blue indicates negatively charged hydrophilic residues, and green indicates neutral hydrophilic residues. The potential interactions involving the GxxxG motifs are boxed in with dotted lines and the possible charge-charge interactions are indicated with dotted lines. The figure is adapted from Rogne, P., 2009 (*85*).

The results in Tables 4.6 and 4.7 suggest that the G_5xxxG_9 motif in PlnE is probably not part of a helix-helix interacting GxxxG motif between PlnE and PlnF. This conclusion is mainly based on the results obtained with the glycine residue in position 5 (G5), which does not seem to be surrounded by any spatial constraints. When G5 was substituted with large hydrophobic residues (leucine and isoleucine), the bacteriocin activity was only marginally reduced (2-6 fold). The results also indicate that G5 is located in a hydrophobic environment, since replacement with a large hydrophilic residue (glutamine) was (in contrast to replacements with leucine and isoleucine) very detrimental (about 100 fold reduction in activity). Replacement with a large positively charged residue (lysine) was, however, relatively well tolerated (about 10 fold

reduction; see below at the end of next page for a possible explanation why lysine has a less detrimental effect than glutamine). Substituting G5 with serine or alanine residues resulted in activity almost as good as, or better than the wild type activity.

In contrast to G5, the glycine residue at position 9 (G9) seems sterically restricted, since substituting G9 with a large hydrophobic (leucine) residue and especially with a large hydrophilic (glutamine) residue was highly detrimental (although the positively charged lysine residue was, again, more tolerated than the glutamine residue). This suggests that the peptide backbone is, at this position, in close contact with another peptide/protein – possibly PlnF and/or a plantaricin EF docking protein. It also seems that G9 is positioned in a hydrophobic environment, since replacement of G9 with the small hydrophobic residue alanine was less detrimental than replacement with the small hydrophilic residue serine (about 10 fold reduction in activity compared to 60-70 fold reduction). These results seem to be in a somewhat 'contrast' to what was observed with the four glycine residues in two helix-helix interacting GxxxG motifs in lactococcin G (*72*): both large hydrophobic and large hydrophilic residues resulted in a quite similar detrimental effect on activity (as was also observed in this study for G9 in PlnE), whereas the glycine to alanine and serine mutations caused a relatively low reduction in activity (*72*). It was thus suggested that it was the size of the side chain residues that was the major cause of reduced activity, rather than the polarity of the residues (*72*). The glycine residue at position 9 in the PlnE peptide, however, seems to be highly affected by the size of the side chains, but also (although to a lesser extent) by the polarity of the side chains.

The replacement of G5 and G9 with the large and hydrophilic, positively charged lysine residue was less detrimental than replacement with the somewhat smaller, but neutral glutamine residue. An explanation could be that the presumably negative impact the lysine residue may have on the structuring of PlnE is partly counteracted by the conceivably positive effect it might have on the initial binding of PlnE to the negatively charged bacterial membrane. Indeed, mutations in the pediocin-like bacteriocin sakacin P, which increased the net positive charge by one, resulted in an increased binding to the target-cell membrane and the antimicrobial activity was about the same as or better than that of wild type sakacin P (*107*).

The results obtained do not discriminate between the two putative models in Fig 5.1, but they do not support the third proposed model, in which the $G_{30}xxxG_{34}$ motif in PlnF interacts with the G_5xxxG_9 motif in PlnE in a parallel manner, since the glycine residue at position 5 is not sterically restricted − a property not compatible with the formation of favourable contact between two transmembrane α-helices.

5.3 Future Aspects

A total of 21 PlnF variants, in which tryptophan and tyrosine residues and the glycine residues in the $G_{30}xxxG_{34}$ motif are substituted, have been constructed in our lab. Purification and activity measurements of these PlnF variants as well as all the mutant PlnE peptides that were constructed in this study are in progress. Determining the effect all of these mutations have on the bacteriocin activity should give a more complete picture of the interaction between PlnE and PlnF as well as the orientation of the plantaricin EF complex in target-cell membranes. Especially the effect of mutations in the $G_{20}xxxG_{24}$ motif in PlnE and the $G_{30}xxxG_{34}$ motif in PlnF should discriminate between the two models presented in Fig 5.1. If individual replacements with large residues in these two motifs, and especially if combinations of mutated variants of these motifs, are highly detrimental, then this will be an indication that these motifs are involved in an interpeptide helix-helix interaction, in support of the model presented in Fig 5.1 A.

Because of the uncertainties involving the estimated amount of mutant PlnE peptides and their calculated concentrations, all the mutant peptides constructed for the G_5xxxG_9 motif should be purified and assayed for confirmation of results obtained in this study.

6. Appendix

6.1 Amino Acids

Single and three letter abbreviation for the amino acids:

Symbol	Abbreviation	Amino Acid
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Tyr	Tyrosine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

6.2 Chemicals, Kits and Equipment

Chemicals and manufacturers:

Chemicals	Manufacturer
Agar	Merck
Agarose	Merck
Ampicillin	CALBIOCHEM
ATP	Amersham Biosciences
100 bp DNA ladder	Fermentas
100 bp DNA ladder plus	Fermentas
$CaCl_2$	Merck
Chloramphenicol	Sigma
dNTPs (dATP, dCTP, dGTP, dTTP)	Pharmacia Biotech
*Dpn*I	Fermentas
Erythromycin	Sigma
Ethidium bromide	Amersham Biosciences
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Glacial acetic acid	KEBO
Glucose	Sigma
Glycerol	VWR
Glycine	Sigma
1 kb DNA ladder	Fermentas
6xLoading Dye Solution	Fermentas
$MgCl_2$	AppliChem
$MgSO_4$	Fermentas
MRS broth	OXOID
M17 broth	OXOID
NaCl	VWR

NaH_2PO_4	PROLABO
Na_2HPO_4	Merck
Peptone	Sigma-Aldrich
Pfu DNA Polymerase buffer	Fermentas
Pfu DNA Polymerase	Fermentas
PfuTurbo DNA Polymerase	Stratagene
Polyethylene glycol (PEG)	Merck
2-propanol	Sigma-Aldrich
SP-Sepharose Fast Flow	GE Healthcare
Taq DNA Polymerase buffer	Fermentas
Taq DNA Polymerase	Fermentas
Trifluoroacetic acid (TFA)	Merck
Tris base	Angus Buffers and Biochemicals
Yeast Extract	Merck

Kits and manufacturers:

Kits	**Manufacturer**
illustra GFX™ PCR DNA and Gel Band Purification Kit	GE Healthcare
DNeasy® Tissue Kit	QIAGEN
NucleoSpin® Tissue Kit	Macherey-Nagel

Equipment and manufacturers:

Equipment	Manufacturer
ÄKTA purifier system	Amersham Biosciences
Beckman Avanti centrifuge J-25	Beckman
Beckman centrifuge J2-MC	Beckman
Electroporation cuvettes (2 mm gap)	Genetronics
Gene Pulser electroporator	BIO-RAD
JA-10 Beckman rotor	Beckman
JA-14 Beckman rotor	Beckman
MR700 Microplate Reader	Dynatech
NanoDrop ND-1000	NanoDrop Technologies
0.20 µm non-pyrogenic sterile filter	Sarstedt
PCR cycler PTC-200	MJ Research
Quartz cuvettes, 1 cm	Hellma
RESOURCE^{TM} RPC column (3 ml)	GE Healthcare
SMART system	Amersham Biosciences
UV Shimadzu UV-160A spectrophotometer	Shimadzu
µRPC C_2/C_{18} SC2.1/10 column	Amersham Biosciences

6.3 Recipes

Agarose gel and gel electrophoresis buffer:

AGAROSE GEL	
50X TAE buffer, 1 L:	**1% Agarose gel:**
254 g Tris base 57.1 ml glacial acetic acid 100 ml 0.1 M EDTA (pH 8) dH_2O to a final volume of 1 L Autoclaved at 121 °C for 20 min	0.4 g agarose 50 ml 1X TAE 3 µl EtBr was applied after cooling the solution to less than 60 °C

Various media for different bacteria:

MEDIA

LB medium, 0.5 L:

5 g NaCl
5 g peptone
2.5 g yeast extract

Dissolved in dH_2O to a final volume of 500 ml. Autoclaved at 121 °C for 20 min.

LB agar plates, 0.5 L:

5 g NaCl
5 g peptone
2.5 g yeast extract
10 g agar

Dissolved in dH_2O to a final volume of 500 ml. Autoclaved at 121 °C for 20 min. Addition of the appropriate antibiotic was applied after cooling the solution to less than 60 °C.

GM17 medium:

18.6 g M17 broth

Dissolved in dH_2O to a final volume of 500 ml. Autoclaved at 112 °C for 10 min. Addition of Tween 80 to a final concentration of 0.1 % (v/v) and glucose to a final concentration of 0.4 % (v/v).

MRS medium, 0.5 L:

26 g MRS broth

Dissolved in dH_2O to a final volume of 500 ml. Autoclaved at 112 °C for 10 min.

MRS agar plates, 0.5 L:

26 g MRS broth
7.5 g agar

Dissolved in dH_2O to a final volume of 500 ml. Autoclaved at 112 °C for 10 min. Addition of the appropriate antibiotic was applied after cooling the solution to less than 60 °C.

MRSSM medium, 0.1 L:

5.2 g MRS broth
17.1 g sucrose (0.5 M)
2 g $MgCl_2$ (0.1 M)

Dissolved in dH2O to a final volume of 100 ml. Sterilized by filtrating through a 0.2 µm non-pyrogenic sterile filter.

Phosphate and elution buffers in cation-exchange chromatography:

BUFFERS

Phosphate buffer, 1 L:
2.48 g NaH_2PO_4
0.26 g Na_2HPO_4
dH_2O to 1 L

Elution buffer, 1 L:
2.48 g NaH_2PO_4
0.26 g Na_2HPO_4
58.44 g NaCl
200 ml 2-propanol
dH_2O to 1 L

6.4 Primers

6.4.1 PCR and Sequencing Primers

PCR and sequencing primers:

Primer[a)]	**Primer Sequence**
SakPB[b)]:	5′-acactttatgcttccggctcgtatgttgtgt-3′
PlnEA[b)]:	5′-ccaaagttataaccgccccgattaaatccacctgtaattg ctgttacttc-3′
PlnEC[b)]:	5′-ccatataaactaaaaaggctgagcttaacgaatacttttc aaaataccacg-3′
PlnEFimm[b)]:	5′-gcacagtaatcgatcgtaaagtatattagcatagc-3′
PlnEFimmstart[b)]:	5′-cgttaagctcagccttttagtttatatgg-3′
pLPV111sekF[b,c)]:	5′-ccagctggcgaaaggggatgtgctgcaagg-3′
pLPV111sekR[b,c)]:	5′-gcaattaatgtgagttagctcactcattagg-3′
T7[d)]:	5′-taatacgactcactataggg-3′
SP6[d)]:	5′-tatttaggtgacactatag-3′

[a)] All primers are produced by Eurogentec S.A. except the primers T7 and SP6 which are produced by Promega.
[b)] The primer was put to use both in PCR and DNA sequencing.
[c)] F = forward primer, R = reverse primer
[d)] Sequencing primers used for verification of insert into the pGEM®-T Easy Vector.

6.4.2 Mutagenic Primers

Mutagenic primers and their primer sequences:

Primer[a)]	Primer Sequence[b)]
G_5xxxG_9 Motif	
PlnE (G5A) F:	5′-ggatttaatcggggcgCttataactttgg-3′
PlnE (G5A) R:	3′-cctaaattagccccgcGaatattgaaacc-5′
PlnE (G5I) F:	5′-ggatttaatcggggcATttataactttgg-3′
PlnE (G5I) R:	3′-cctaaattagccccgTAaatattgaaacc-5′
PlnE (G5K) F:	5′-ggatttaatcggggcAAAtataactttgg-3′
PlnE (G5K) R:	3′-cctaaattagccccgTTTatattgaaacc-5′
PlnE (G5L) F:	5′-ggatttaatcggggcTTAtataactttgg-3′
PlnE (G5L) R:	3′-cctaaattagccccgAATatattgaaacc-5′
PlnE (G5Q) F:	5′-ggatttaatcggggcCAAtataactttgg-3′
PlnE (G5Q) R:	3′-cctaaattagccccgGTTatattgaaacc-5′
PlnE (G5S) F:	5′-ggatttaatcggggcAgttataactttgg-3′
PlnE (G5S) R:	3′-cctaaattagccccgTcaatattgaaacc-5′
PlnE (G9A) F:	5′-ggttataactttgCtaaaagtgttcgac-3′
PlnE (G9A) R:	3′-ccaatattgaaacGattttcacaagctg-5′
PlnE (G9I) F:	5′-ggttataactttATtaaaagtgttcgac-3′
PlnE (G9I) R:	3′-ccaatattgaaaTAattttcacaagctg-5′
PlnE (G9K) F:	5′-ggttataactttAAAaaaagtgttcgac-3′
PlnE (G9K) R:	3′-ccaatattgaaaTTTttttcacaagctg-5′
PlnE (G9L) F:	5′-ggttataactttTTAaaaagtgttcgac-3′
PlnE (G9L) R:	3′-ccaatattgaaaAATttttcacaagctg-5′
PlnE (G9Q) F:	5′-ggttataactttCAAaaaagtgttcgac-3′
PlnE (G9Q) R:	3′-ccaatattgaaaGTTttttcacaagctg-5′
PlnE (G9S) F:	5′-ggttataactttAgtaaaagtgttcgac-3′
PlnE (G9S) R:	3′-ccaatattgaaaTcattttcacaagctg-5′
$G_{20}xxxG_{24}$ Motif	
PlnE (G20A) F:	5′-gttgatgcaattgCttcagttgcaggcattcg-3′
PlnE (G20A) R:	3′-caactacgttaacGaagtcaacgtccgtaagc-5′
PlnE (G20I) F:	5′-gttgatgcaattATttcagttgcaggcattcg-3′
PlnE (G20I) R:	3′-caactacgttaaTAaagtcaacgtccgtaagc-5′

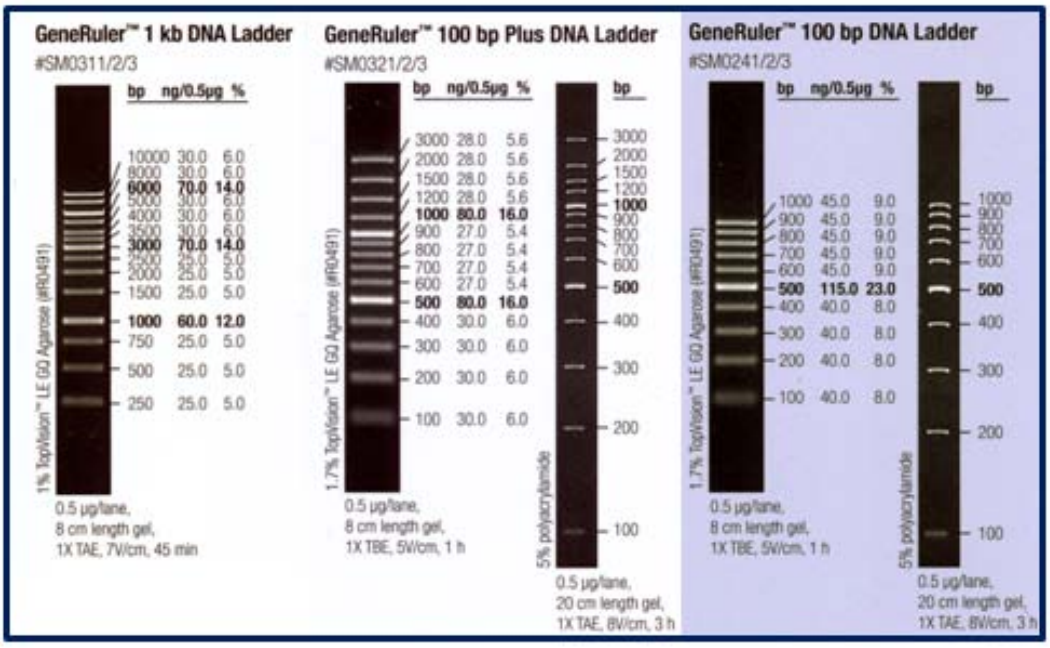
PlnE (G20K) F:	5′-gttgatgcaattAAAtcagttgcaggcattcg-3′
PlnE (G20K) R:	3′-caactacgttaaTTTagtcaacgtccgtaagc-5′
PlnE (G20L) F:	5′-gttgatgcaattTTAtcagttgcaggcattcg-3′
PlnE (G20L) R:	3′-caactacgttaaAATagtcaacgtccgtaagc-5′
PlnE (G20Q) F:	5′-gttgatgcaattCAAtcagttgcaggcattcg-3′
PlnE (G20Q) R:	3′-caactacgttaaGTTagtcaacgtccgtaagc-5′
PlnE (G20S) F:	5′-gttgatgcaattAgttcagttgcaggcattcg-3′
PlnE (G20S) R:	3′-caactacgttaaTcaagtcaacgtccgtaagc-5′
PlnE (G24A) F:	5′-ggttcagttgcagCAattcgtggtattttg-3′
PlnE (G24A) R:	3′-ccaagtcaacgtcGTtaagcaccataaaac-5′
PlnE (G24I) F:	5′-ggttcagttgcaATTattcgtggtattttg-3′
PlnE (G24I) R:	3′-ccaagtcaacgtTAAtaagcaccataaaac-5′
PlnE (G24K) F:	5′-ggttcagttgcaAAAattcgtggtattttg-3′
PlnE (G24K) R:	3′-ccaagtcaacgtTTTtaagcaccataaaac-5′
PlnE (G24L) F:	5′-ggttcagttgcaTTAattcgtggtattttg-3′
PlnE (G24L) R:	3′-ccaagtcaacgtAATtaagcaccataaaac-5′
PlnE (G24Q) F:	5′-ggttcagttgcaCAAattcgtggtattttg-3′
PlnE (G24Q) R:	3′-ccaagtcaacgtGTTtaagcaccataaaac-5′
PlnE (G24S) F:	5′-ggttcagttgcaAgTattcgtggtattttg-3′
PlnE (G24S) R:	3′-ccaagtcaacgtTcAtaagcaccataaaac-5′
Tyrosine Residue	
PlnE (Y6F) F:	5′-atcggggcggttTtaactttggtaaaag-3′
PlnE (Y6F) R:	3′-tagccccgccaaAattgaaaccattttc-5′
PlnE (Y6L) F:	5′-atcggggcggttTAaactttggtaaaag-3′
PlnE (Y6L) R:	3′-tagccccgccaaATttgaaaccattttc-5′
PlnE (Y6R) F:	5′-atcggggcggtAGAaactttggtaaaag-3′
PlnE (Y6R) R:	3′-tagccccgccaTCTttgaaaccattttc-5′
PlnE (Y6W) F:	5′-atcggggcggttGGaactttggtaaaag-3′
PlnE (Y6W) R:	3′-tagccccgccaaCCttgaaaccattttc-5′

[a] F = forward primer, R = reverse primer. All mutagenic primers are produced by Eurogentec S.A.

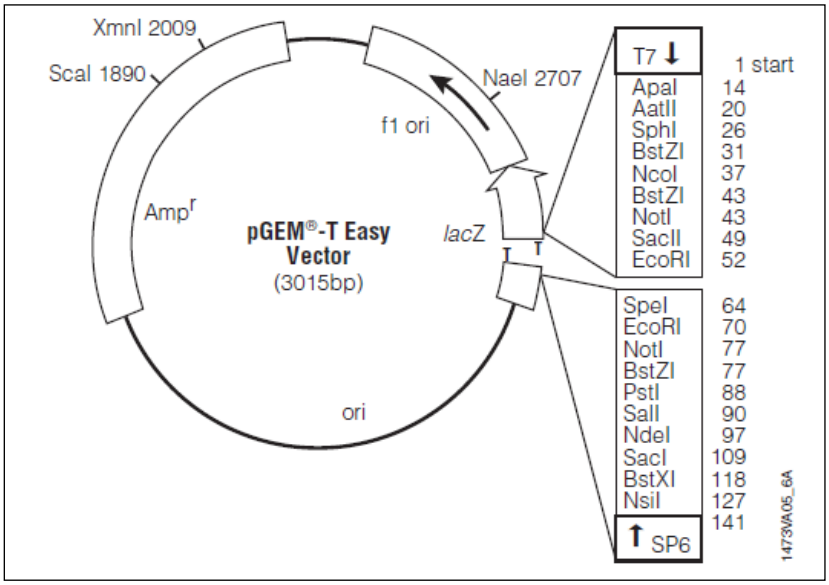
[b] Capital letters indicate the mutagenic positions. The three-letter codon of the new amino acid residue is marked in green.

6.5 Sizing Ladders

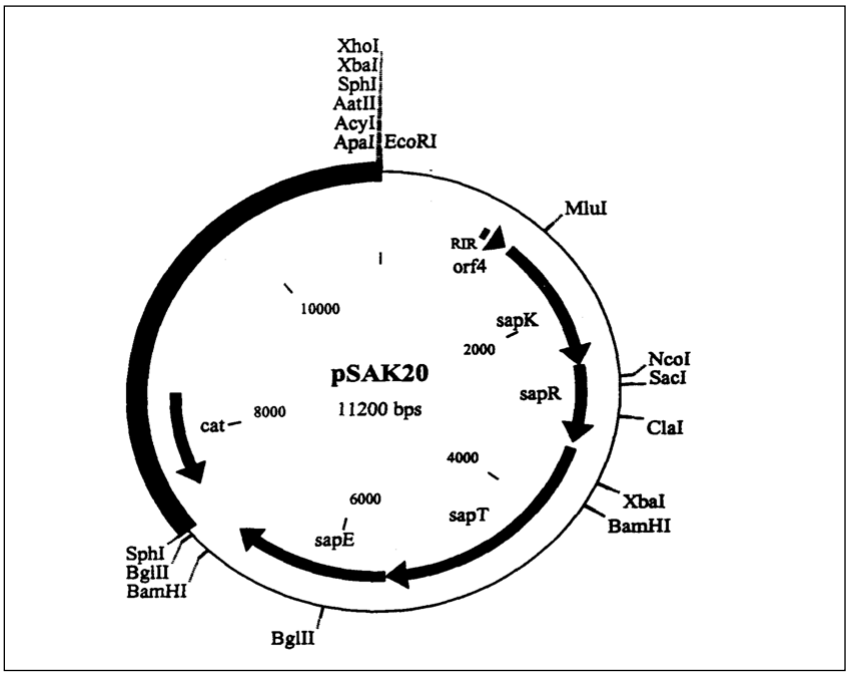
1 kb DNA Ladder: **100 bp Plus DNA Ladder:** **100 bp DNA Ladder:**



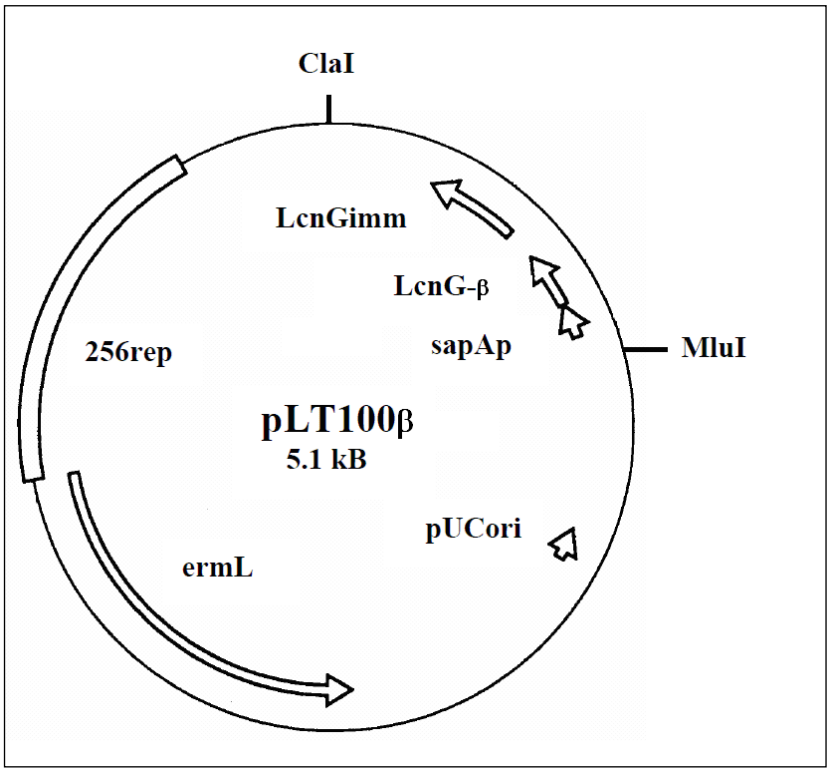
6.6 Plasmids



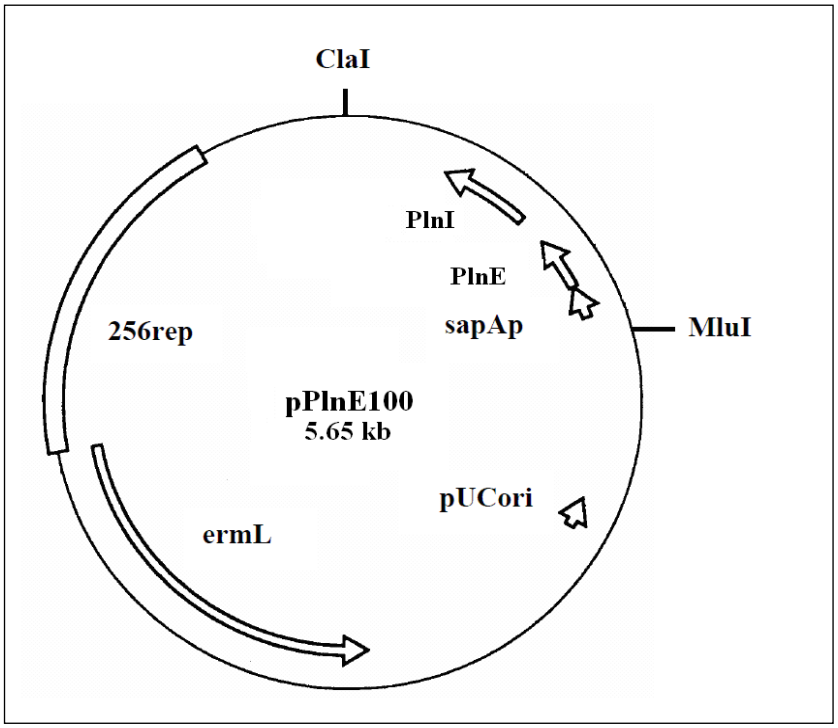
A circle map of the pGEM®-T Easy Vector. The vector contains a marker for ampicillin-resistance (*Amp*^{r}) and the gene *lacZ*. The gene encodes the β-galactosidase enzyme allowing for blue-white selection. The figure is adapted from Promega (*96*).



A plasmid chart of pSAK20. The plasmid contains a marker for chloramphenicol-resistance (*cat*) as well as the operon *orf4-sapKRTE*, which is responsible for the transcriptional regulation, processing and secretion of sakacin A. *orf4-sapKR* constitute a three-component regulatory system (*100*).The gene orf4 encodes a peptide pheromone, *sapK* encodes a histidine kinase, *sapR* encodes a response regulator, *sapT* encodes an ABC transporter and *sapE* encodes an accessory factor (*99*).



A plasmid chart of pLT100β. A pLPV111 derivative containing a marker for erythromycin-resistance (*ermL*), the gene encoding LcnG-β, one of the peptides constituting the two-peptide bacteriocin lactococcin G, and the gene encoding the lactococcin G immunity protein (LcnGimm). The gene encoding LcnG-β is fused to the sakacin P leader sequence and the sakacin A promoter (sapAp). The figure is adapted from Oppegård, C., 2005 (*110*).



A plasmid chart of pPlnE100. A pLPV111 derivative constructed from pLT100β. The plasmid contains the sakacin A promoter (sapAp), the sakacin P leader sequence fused to the *plnE*-gene followed by the *plnI*-gene. The plasmid also contains a marker for erythromycin-resistance (*ermL*).

7. References

1. Hancock, R. E. W., and Diamond, G. (2000) The Role of Cationic Antimicrobial Peptides in Innate Host Defences, *Trends Microbiol. 8*, 402-410.
2. Nissen-Meyer, J., and Nes, I. F. (1997) Ribosomally Synthesized Antimicrobial Peptides: Their Function, Structure, Biogenesis and Mechanism of Action, *Arch. Microbiol. 167*, 67-77.
3. Fimland, G., and Nissen-Meyer, J. (2007) Genetically Modified Bacteriocins, In *Bacteriocins: Current Research and Applications* (Riley, M. A., and Gillor, O., Eds.), pp 43-66, Horizon Bioscience, Wymondham, Norfolk.
4. Hancock, R. E. W. (2001) Cationic Peptides: Effectors in Innate Immunity and Novel Antimicrobials, *Lanc. Infect. Dis. 1*, 156-164.
5. Boman, H. G. (1996) Peptide Antibiotics: Holy or Heretic Grails of Innate Immunity?, *Scand. J. Immunol. 43*, 475-482.
6. Zasloff, M. (2002) Antimicrobial Peptides of Multicellular Organisms, *Nature 415*, 389-395.
7. Auvynet, C., and Rosenstein, Y. (2009) Multifunctional Host Defense Peptides: Antimicrobial Peptides, the Small yet Big Players in Innate and Adaptive Immunity, *FEBS J. 276*, 6497-6508.
8. Yeaman, M. R., and Yount, N. Y. (2003) Mechanisms of Antimicrobial Peptide Action and Resistance, *Pharmacol. Rev. 55*, 27-55.
9. Hancock, R. E. W., and Sahl, H. G. (2006) Antimicrobial and Host-Defense Peptides as new Anti-Infective Therapeutic Strategies, *Nature Biotechnol. 24*, 1551-1557.
10. Nissen-Meyer, J., Rogne, P., Oppegård, C., Haugen, H. S., and Kristiansen, P. E. (2009) Structure-Function Relationships of the Non-Lanthionine-Containing Peptide (class II) Bacteriocins Produced by Gram-Positive Bacteria, *Curr. Pharm. Biotech. 10*, 19-37.
11. Gratia, J.-P. (2000) André Gratia: A Forerunner in Microbial and Viral Genetics, *Genetics 156*, 471-476.
12. Riley, M. A. (1998) Molecular Mechanisms of Bacteriocin Evolution, *Annu. Rev. Genet. 32*, 255-278.
13. Savadogo, A., Ouattara, C. A. T., Bassole, I. H. N., and Alfred, S. A. (2006) Bacteriocins and Lactic Acid Bacteria - a Minireview, *Afr. J. Biotechnol. 5*, 678-683.
14. Leroy, F., and De Vuyst, L. (2004) Lactic Acid Bacteria as Functional Starter Cultures for the Food Fermentation Industry, *Trends Food Sci. Tech. 15*, 67-78.
15. Kleerebezem, M., Hols, P., Bernard, E., Rolain, T., Zhou, M. M., Siezen, R. J., and Bron, P. A. (2010) The Extracellular Biology of the Lactobacilli, *FEMS Microbiol. Rev. 34*, 199-230.

16. Delves-Broughton, J., Blackburn, P., Evans, R. J., and Hugenholtz, J. (1996) Applications of the Bacteriocin Nisin, *Antonie van Leeuwenhoek 69*, 193-202.

17. Sobrino-Lopez, A., and Martin-Belloso, O. (2008) Use of Nisin and Other Bacteriocins for Preservation of Dairy Products, *Int. Dairy J. 18*, 329-343.

18. Cotter, P. D., Hill, C., and Ross, R. P. (2005) Bacteriocins: Developing Innate Immunity for Food, *Nat. Rev. Microbiol. 3*, 777-788.

19. Corr, S. C., Li, Y., Riedel, C. U., O'Toole, P. W., Hill, C., and Gahan, C. G. M. (2007) Bacteriocin Production as a Mechanism for the Antiinfective Activity of *Lactobacillus salivarius* UCC118, *Proc. Natl. Acad. Sci. USA 104*, 7617-7621.

20. Wachsman, M. B., Castilla, V., Holgado, A. P. D., de Torres, R. A., Sesma, F., and Coto, C. E. (2003) Enterocin CRL35 Inhibits Late Stages of HSV-1 and HSV-2 Replication *in vitro*, *Antivir. Res. 58*, 17-24.

21. Willey, J. M., and van der Donk, W. A. (2007) Lantibiotics: Peptides of Diverse Structure and Function, *Annu. Rev. Microbiol. 61*, 477-501.

22. Garneau, S., Martin, N. I., and Vederas, J. C. (2002) Two-Peptide Bacteriocins Produced by Lactic Acid Bacteria, *Biochimie 84*, 577-592.

23. Drider, D., Fimland, G., Hechard, Y., McMullen, L. M., and Prevost, H. (2006) The Continuing Story of Class IIa Bacteriocins, *Microbiol. Mol. Biol. Rev. 70*, 564-582.

24. Oppegård, C., Rogne, P., Emanuelsen, L., Kristiansen, P. E., Fimland, G., and Nissen-Meyer, J. (2007) The Two-Peptide Class II Bacteriocins: Structure, Production, and Mode of Action, *J. Mol. Microbiol. Biotechnol. 13*, 210-219.

25. Diep, D. B., Straume, D., Kjos, M., Torres, C., and Nes, I. F. (2009) An Overview of the Mosaic Bacteriocin *pln* Loci from *Lactobacillus plantarum*, *Peptides 30*, 1562-1574.

26. Nissen-Meyer, J., Holo, H., Håvarstein, L. S., Sletten, K., and Nes, I. F. (1992) A Novel Lactococcal Bacteriocin Whose Activity Depends on the Complementary Action of Two Peptides, *J. Bacteriol. 174*, 5686-5692.

27. Nissen-Meyer, J., Oppegård, C., Rogne, P., Haugen, H. S., and Kristiansen, P. E. (2010) Structure and Mode-of-Action of the Two-Peptide (Class-IIb) Bacteriocins, *Probiotics Antimicrob. Prot. 2*, 52-60.

28. Zendo, T., Koga, S., Shigeri, Y., Nakayama, J., and Sonomoto, K. (2006) Lactococcin Q, a Novel Two-Peptide Bacteriocin Produced by *Lactococcus lactis* QU 4, *Appl. Environ. Microbiol. 72*, 3383-3389.

29. Franz, C. M. A. P., Grube, A., Herrmann, A., Abriouel, H., Starke, J., Lombardi, A., Tauscher, B., and Holzapfel, W. H. (2002) Biochemical and Genetic Characterization of the Two-Peptide Bacteriocin Enterocin 1071 Produced by *Enterococcus faecalis* FAIR-E 309, *Appl. Environ. Microbiol. 68*, 2550-2554.

30. Balla, E., Dicks, L. M. T., Du Toit, M., van der Merwe, M. J., and Holzapfel, W. H. (2000) Characterization and Cloning of the Genes Encoding Enterocin 1071A and Enterocin 1071B, Two Antimicrobial Peptides Produced by *Enterococcus faecalis* BFE 1071, *Appl. Environ. Microbiol. 66*, 1298-1304.

31. Balla, E., and Dicks, L. M. T. (2005) Molecular Analysis of the Gene Cluster Involved in the Production and Secretion of Enterocins 1071A and 1071B and of the Genes Responsible for the Replication and Transfer of Plasmid pEF1071, *Int. J. Food Microbiol. 99*, 33-45.

32. Maldonado-Barragan, A., Caballero-Guerrero, B., Jimenez, E., Jimenez-Diaz, R., Ruiz-Barba, J. L., and Rodriguez, J. M. (2009) Enterocin C, a Class IIb Bacteriocin Produced by *E. faecalis* C901, a Strain Isolated from Human Colostrum, *Int. J. Food Microbiol. 133*, 105-112.

33. Hu, C. B., Malaphan, W., Zendo, T., Nakayama, J., and Sonomoto, K. (2010) Enterocin X, a Novel Two-Peptide Bacteriocin from *Enterococcus faecium* KU-B5 has an Antibacterial Spectrum Entirely Different from Those of Its Component Peptides, *Appl. Environ. Microbiol. 76*, 4542-4545.

34. Anderssen, E. L., Diep, D. B., Nes, I. F., Eijsink, V. G. H., and Nissen-Meyer, J. (1998) Antagonistic Activity of *Lactobacillus plantarum* C11: Two New Two-Peptide Bacteriocins, Plantaricins EF and JK, and the Induction Factor Plantaricin A, *Appl. Environ. Microbiol. 64*, 2269-2272.

35. Diep, D. B., Håvarstein, L. S., and Nes, I. F. (1996) Characterization of the Locus Responsible for the Bacteriocin Production in *Lactobacillus plantarum* C11, *J. Bacteriol. 178*, 4472-4483.

36. Jimenez-Diaz, R., Ruiz-Barba, J. L., Cathcart, D. P., Holo, H., Nes, I. F., Sletten, K. H., and Warner, P. J. (1995) Purification and Partial Amino Acid Sequence of Plantaricin S, a Bacteriocin Produced by *Lactobacillus plantarum* LPCO10, the Activity of which Depends on the Complementary Action of Two Peptides, *Appl. Environ. Microbiol. 61*, 4459-4463.

37. Stephens, S. K., Floriano, B., Cathcart, D. P., Bayley, S. A., Witt, V. F., Jimenez-Diaz, R., Warner, P. J., and Ruiz-Barba, J. L. (1998) Molecular Analysis of the Locus Responsible for Production of Plantaricin S, a Two-Peptide Bacteriocin Produced by *Lactobacillus plantarum* LPCO10, *Appl. Environ. Microbiol. 64*, 1871-1877.

38. Maldonado, A., Ruiz-Barba, J. L., and Jimenez-Diaz, R. (2003) Purification and Genetic Characterization of Plantaricin NC8, a Novel Coculture-Inducible Two-Peptide Bacteriocin from *Lactobacillus plantarum* NC8, *Appl. Environ. Microbiol. 69*, 383-389.

39. Cuozzo, S. A., Sesma, F., Palacios, J. M., Holgado, A. P. D., and Raya, R. R. (2000) Identification and Nucleotide Sequence of Genes Involved in the Synthesis of Lactocin 705, a Two-Peptide Bacteriocin from *Lactobacillus casei* CRL 705, *FEMS Microbiol. Lett. 185*, 157-161.

40. Allison, G. E., Fremaux, C., and Klaenhammer, T. R. (1994) Expansion of Bacteriocin Activity and Host-Range Upon Complementation of two Peptides Encoded within the Lactacin F Operon, *J. Bacteriol. 176*, 2235-2241.

41. Fremaux, C., Ahn, C., and Klaenhammer, T. R. (1993) Molecular Analysis of the Lactacin F Operon, *Appl. Environ. Microbiol. 59*, 3906-3915.

42. McCormick, J. K., Poon, A., Sailer, M., Gao, Y., Roy, K. L., McMullen, L. M., Vederas, J. C., Stiles, M. E., and Van Belkum, M. J. (1998) Genetic Characterization and Heterologous Expression of Brochocin-C, an Antibotulinal, Two-Peptide Bacteriocin Produced by *Brochothrix campestris* ATCC 43754, *Appl. Environ. Microbiol. 64*, 4757-4766.

43. Marciset, O., Jeronimus-Stratingh, M. C., Mollet, B., and Poolman, B. (1997) Thermophilin 13, a Nontypical Antilisterial Poration Complex Bacteriocin that Functions without a Receptor, *J. Biol. Chem. 272*, 14277-14284.

44. Flynn, S., van Sinderen, D., Thornton, G. M., Holo, H., Nes, I. F., and Collins, J. K. (2002) Characterization of the Genetic Locus Responsible for the Production of ABP-118, a Novel Bacteriocin Produced by the Probiotic Bacterium *Lactobacillus salivarius* subsp. *salivarius* UCC118, *Microbiology 148*, 973-984.

45. Barrett, E., Hayes, M., O'Connor, P., Gardiner, G., Fitzgerald, G. F., Stanton, C., Ross, R. P., and Hill, C. (2007) Salivaricin P, One of a Family of Two-Component Antilisterial Bacteriocins Produced by Intestinal Isolates of *Lactobacillus salivarius*, *Appl. Environ. Microbiol. 73*, 3719-3723.

46. Qi, F. X., Chen, P., and Caufield, P. W. (2001) The Group I Strain of *Streptococcus mutans*, UA140, Produces Both the Lantibiotic Mutacin I and a Nonlantibiotic Bacteriocin, Mutacin IV, *Appl. Environ. Microbiol. 67*, 15-21.

47. Ghrairi, T., Frere, J., Berjeaud, J. M., and Manai, M. (2005) Lactococcin MMT24, a Novel Two-Peptide Bacteriocin Produced by *Lactococcus lactis* Isolated from Rigouta Cheese, *Int. J. Food Microbiol. 105*, 389-398.

48. van Belkum, M. J., Hayema, B. J., Jeeninga, R. E., Kok, J., and Venema, G. (1991) Organization and Nucleotide-Sequences of two Lactococcal Bacteriocin Operons, *Appl. Environ. Microbiol. 57*, 492-498.

49. Blom, H., Katla, T., Holck, A., Sletten, K., Axelsson, L., and Holo, H. (1999) Characterization, Production and Purification of Leucocin H, a Two-Peptide Bacteriocin from *Leuconostoc* MF215B, *Curr. Microbiol. 39*, 43-48.

50. Sáenz, Y., Rojo-Bezares, B., Navarro, L., Diez, L., Somalo, S., Zarazaga, M., Ruiz-Larrea, F., and Torres, C. (2009) Genetic Diversity of the *pln L*ocus among Oenological *Lactobacillus plantarum* Strains, *Int. J. Food Microbiol. 134*, 176-183.

51. Håvarstein, L. S., Diep, D. B., and Nes, I. F. (1995) A Family of Bacteriocin ABC Transporters Carry out Proteolytic Processing of Their Substrates Concomitant with Export, *Mol. Microbiol. 16*, 229-240.

52. Oppegård, C., Emanuelsen, L., Thorbek, L., Fimland, G., and Nissen-Meyer, J. (2010) The Lactococcin G Immunity Protein Recognizes Specific Regions in Both Peptides Constituting the Two-Peptide Bacteriocin Lactococcin G, *Appl. Environ. Microbiol. 76*, 1267-1273.

53. Nes, I. F., Diep, D. B., Håvarstein, L. S., Brurberg, M. B., Eijsink, V., and Holo, H. (1996) Biosynthesis of Bacteriocins in Lactic Acid Bacteria, *Antonie van Leeuwenhoek 70*, 113-128.

54. Diep, D. B., Myhre, R., Johnsborg, O., Aakra, A., and Nes, I. F. (2003) Inducible Bacteriocin Production in *Lactobacillus* is Regulated by Differential Expression of the *pln* Operons and by Two Antagonizing Response Regulators, the Activity of which is Enhanced upon Phosphorylation, *Mol. Microbiol. 47*, 483-494.

55. Eijsink, V. G. H., Axelsson, L., Diep, D. B., Håvarstein, L. S., Holo, H., and Nes, I. F. (2002) Production of Class II Bacteriocins by Lactic Acid Bacteria; an Example of Biological Warfare and Communication, *Antonie van Leeuwenhoek 81*, 639-654.

56. Kleerebezem, M., and Quadri, L. E. (2001) Peptide Pheromone-Dependent Regulation of Antimicrobial Peptide Production in Gram-Positive Bacteria: a Case of Multicellular Behavior, *Peptides 22*, 1579-1596.

57. Straume, D., Kjos, M., Nes, I. F., and Diep, D. B. (2007) Quorum-Sensing Based Bacteriocin Production is Down-Regulated by N-Terminally Truncated Species of Gene Activators, *Mol. Genet. Genomics 278*, 283-293.

58. Hauge, H. H., Mantzilas, D., Eijsink, V. G. H., and Nissen-Meyer, J. (1999) Membrane-Mimicking Entities Induce Structuring of the Two-Peptide Bacteriocins Plantaricin E/F and Plantaricin J/K, *J. Bacteriol. 181*, 740-747.

59. Hauge, H. H., Mantzilas, D., Moll, G. N., Konings, W. N., Driessen, A. J. M., Eijsink, V. G. H., and Nissen-Meyer, J. (1998) Plantaricin A is an Amphiphilic α-Helical Bacteriocin-like Pheromone which Exerts Antimicrobial and Pheromone Activities Through Different Mechanisms, *Biochemistry 37*, 16026-16032.

60. Hauge, H. H., Nissen-Meyer, J., Nes, I. F., and Eijsink, V. G. H. (1998) Amphiphilic α-Helices are Important Structural Motifs in the α and β Peptides that Constitute the Bacteriocin Lactococcin G - Enhancement of Helix Formation upon α-β Interaction, *Eur. J. Biochem. 251*, 565-572.

61. Fimland, N., Rogne, P., Fimland, G., Nissen-Meyer, J., and Kristiansen, P. E. (2008) Three-Dimensional Structure of the Two Peptides that Constitute the Two-Peptide Bacteriocin Plantaricin EF, *Biochim. Biophys. Acta 1784*, 1711-1719.

62. Rogne, P., Fimland, G., Nissen-Meyer, J., and Kristiansen, P. E. (2008) Three-Dimensional Structure of the Two Peptides that Constitute the Two-Peptide Bacteriocin Lactococcin G, *Biochim. Biophys. Acta 1784*, 543-554.

63. Rogne, P., Haugen, C., Fimland, G., Nissen-Meyer, J., and Kristiansen, P. E. (2009) Three-Dimensional Structure of the Two-Peptide Bacteriocin Plantaricin JK, *Peptides 30*, 1613-1621.

64. Moll, G., Hauge, H. H., Nissen-Meyer, J., Nes, I. F., Konings, W. N., and Driessen, a. A. J. M. (1998) Mechanistic Properties of the Two-Component Bacteriocin Lactococcin G, *J. Bacteriol. 180*, 96-99.

65. Moll, G. N., van den Akker, E., Hauge, H. H., Nissen-Meyer, J., Nes, I. F., Konings, W. N., and Driessen, A. J. M. (1999) Complementary and Overlapping Selectivity of the Two-Peptide Bacteriocins Plantaricin EF and JK, *J. Bacteriol. 181*, 4848-4852.

66. Moll, G., Ubbink-Kok, T., Hauge, H. H., Nissen-Meyer, J., Nes, I. F., Konings, W. N., and Driessen, A. J. M. (1996) Lactococcin G is a Potassium Ion-Conducting, Two-Component Bacteriocin, *J. Bacteriol. 178*, 600-605.

67. Abee, T., Klaenhammer, T. R., and Letellier, L. (1994) Kinetic Studies of the Action of Lactacin-F, a Bacteriocin Produced by *Lactobacillus johnsonii* that forms Poration Complexes in the Cytoplasmic Membrane, *Appl. Environ. Microbiol. 60*, 1006-1013.

68. Castellano, P., Raya, R., and Vignolo, G. (2003) Mode of Action of Lactocin 705, a Two-Component Bacteriocin from *Lactobacillus casei* CRL705, *Int. J. Food Microbiol. 85*, 35-43.

69. Oppegård, C., Fimland, G., Thorbaek, L., and Nissen-Meyer, J. (2007) Analysis of the Two-Peptide Bacteriocins Lactococcin G and Enterocin 1071 by Site-Directed Mutagenesis, *Appl. Environ. Microbiol. 73*, 2931-2938.

70. Hechard, Y., and Sahl, H. G. (2002) Mode of Action of Modified and Unmodified Bacteriocins from Gram-Positive Bacteria, *Biochimie 84*, 545-557.

71. Diep, D. B., Skaugen, M., Salehian, Z., Holo, H., and Nes, I. F. (2007) Common Mechanisms of Target Cell Recognition and Immunity for Class II Bacteriocins, *Proc. Natl. Acad. Sci. USA 104*, 2384-2389.

72. Oppegård, C., Schmidt, J., Kristiansen, P. E., and Nissen-Meyer, J. (2008) Mutational Analysis of Putative Helix-Helix Interacting GxxxG-Motifs and Tryptophan Residues in the Two-Peptide Bacteriocin Lactococcin G, *Biochemistry 47*, 5242-5249.

73. Diep, D. B., Håvarstein, L. S., and Nes, I. F. (1995) A Bacteriocin-like Peptide Induces Bacteriocin Synthesis in *Lactobacillus plantarum* C11, *Mol. Microbiol. 18*, 631-639.

74. Daeschel, M. A., McKenney, M. C., and and McDonald, L. C. (1990) Bacteriocidal Activity of *Lactobacillus plantarum* C-11, *Food Microbiol. 7*, 91-98.

75. Nissen-Meyer, J., Larsen, A. G., Sletten, K., Daeschel, M., and Nes, I. F. (1993) Purification and Characterization of Plantaricin A, a *Lactobacillus plantarum* Bacteriocin Whose Activity Depends on the Action of Two Peptides, *J. Gen. Microbiol. 139*, 1973-1978.

76. Diep, D. B., Håvarstein, L. S., Nissen-Meyer, J., and Nes, I. F. (1994) The Gene Encoding Plantaricin A, a Bacteriocin from *Lactobacillus plantarum* C11, is Located on the Same Transcription Unit as an Agr-Like Regulatory System, *Appl. Environ. Microbiol. 60*, 160-166.

77. Kristiansen, P. E., Fimland, G., Mantzilas, D., and Nissen-Meyer, J. (2005) Structure and Mode of Action of the Membrane-Permeabilizing Antimicrobial Peptide Pheromone Plantaricin A, *J. Biol. Chem. 280*, 22945-22950.

78. Kjos, M., Snipen, L., Salehian, Z., Nes, I. F., and Diep, D. B. (2010) The Abi Proteins and Their Involvement in Bacteriocin Self-Immunity, *J. Bacteriol. 192*, 2068-2076.

79. Diep, D. B., Johnsborg, O., Risøen, P. A., and Nes, I. F. (2001) Evidence for Dual Functionality of the Operon *pln*ABCD in the Regulation of Bacteriocin Production in *Lactobacillus plantarum, Mol. Microbiol. 41,* 633-644.

80. Straume, D., Johansen, R. F., Bjørås, M., Nes, I. F., and Diep, D. B. (2009) DNA Binding Kinetics of Two Response Regulators, PlnC and PlnD, from the Bacteriocin Regulon of *Lactobacillus plantarum* C11, *BMC Biochem. 10,* 17.

81. Senes, A., Engel, D. E., and DeGrado, W. F. (2004) Folding of Helical Membrane Proteins: the Role of Polar, GxxxG-like and Proline Motifs, *Curr. Opin. Struct. Biol. 14*, 465-479.

82. Senes, A., Ubarretxena-Belandia, I., and Engelman, D. M. (2001) The Cα—H···O Hydrogen Bond: A Determinant of Stability and Specificity in Transmembrane Helix Interactions, *Proc. Natl. Acad. Sci. USA 98*, 9056-9061.

83. Javadpour, M. M., Eilers, M., Groesbeek, M., and Smith, S. O. (1999) Helix Packing in Polytopic Membrane Proteins: Role of Glycine in Transmembrane Helix Association, *Biophys. J. 77*, 1609-1618.

84. Schneider, D., and Engelman, D. M. (2004) Motifs of Two Small Residues can Assist but are not Sufficient to Mediate Transmembrane Helix Interactions, *J. Mol. Biol. 343*, 799-804.

85. Rogne, P. (2009) Structure and Function of Two-Peptide Bacteriocins, *Ph.D. thesis, IMBV, Univ. Oslo*.

86. Killian, J. A., and von Heijne, G. (2000) How Proteins Adapt to a Membrane-Water Interface, *Trends Biochem. Sci. 25*, 429-434.

87. Yau, W. M., Wimley, W. C., Gawrisch, K., and White, S. H. (1998) The Preference of Tryptophan for Membrane Interfaces, *Biochemistry 37*, 14713-14718.

88. de Planque, M. R. R., Kruijtzer, J. A. W., Liskamp, R. M. J., Marsh, D., Greathouse, V., Koeppe, R. E., de Kruijff, B., and Killian, J. A. (1999) Different Membrane Anchoring Positions of Tryptophan and Lysine in Synthetic Transmembrane α-Helical Peptides, *J. Biol. Chem. 274*, 20839-20846.

89. Ridder, A. N. J. A., Morein, S., Stam, J. G., Kuhn, A., de Kruijff, B., and Killian, J. A. (2000) Analysis of the Role of Interfacial Tryptophan Residues in Controlling the Topology of Membrane Proteins, *Biochemistry 39*, 6521-6528.

90. Hanahan, D. (1983) Studies on Transformation of *Escherichia coli* with Plasmids, *J. Mol. Biol. 166*, 557-580.

91. Agilent Technologies (2009) QuikChange® Site-Directed Mutagenesis - Instruction Manual.

92. Aukrust, T. W., Brurberg, M. B., and Nes, I. F. (1995) Transformation of *Lactobacillus* by Electroporation, In *Methods in Molecular Biology; Electroporation Protocols for Microorganisms* (Nickoloff, J. A., Ed.), pp 201-208, Humana Press Inc., New Jersey, USA.

93. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Site-directed Mutagenesis by Overlap Extension using the Polymerase Chain Reaction, *Gene 77*, 51-59.

94. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase, *Science 239*, 487-491.

95. Sarkar, G., and Sommer, S. S. (1990) The "Megaprimer" Method of Site-Directed Mutagenesis, *Biotechniques 8*, 404-407.

96. Promega. (2009) pGEM®-T and pGEM®-T Easy Vector Systems - Technical Manual No. 042.

97. Russell, J. S. D. W. (2001) *Molecular Cloning - A Laboratory Manual*, Vol. 1, 3. ed., Cold Spring Harbor Laboratory Press, New York, USA.

98. Axelsson, L., Katla, T., Bjørnslett, M., Eijsink, V. G. H., and Holck, A. (1998) A System for Heterologous Expression of Bacteriocins in *Lactobacillus sake*, *FEMS Microbiol. Lett. 168*, 137-143.

99. Axelsson, L., and Holck, A. (1995) The Genes Involved in Production of and Immunity to Sakacin A, a Bacteriocin from *Lactobacillus sake* Lb706, *J. Bacteriol. 177*, 2125-2137.

100. Diep, D. B., Axelsson, L., Grefsli, C., and Nes, I. F. (2000) The Synthesis of the Bacteriocin Sakacin A is a Temperature-Sensitive Process Regulated by a Pheromone Peptide through a Three-Component Regulatory System, *Microbiology 146*, 2155-2160.

101. Thorbek, L. (2006) Heterolog Ekspresjon av To-Peptid Bakteriosinet Lactococcin G, *Master thesis, IMBV,Univ. Oslo*.

102. Sheehan, D. (2000) *Physical Biochemistry: Principles and Applications*, John Wiley & Sons Ltd., Colchester, UK.

103. Uteng, M., Hauge, H. H., Brondz, I., Nissen-Meyer, J., and Fimland, G. (2002) Rapid Two-Step Procedure for Large-Scale Purification of Pediocin-Like Bacteriocins and Other Cationic Antimicrobial Peptides from Complex Culture Medium, *Appl. Environ. Microbiol. 68*, 952-956.

104. Harris, D. C. (2003) *Quantitative Chemical Analysis*, 6 ed., W. H. Freeman and Company, New york, USA.

105. Kuipers, B. J. H., and Gruppen, H. (2007) Prediction of Molar Extinction Coefficients of Proteins and Peptides using UV Absorption of the Constituent Amino Acids at 214 nm to Enable Quantitative Reverse Phase High-Performance Liquid Chromatography-Mass Spectrometry Analysis, *J. Agr. Food Chem. 55*, 5445-5451.

106. Jørgenrud, B. M. (2009) Construction of a Heterologous Expression Vector for Plantaricin F, One of the Peptides Constituting the Two-Peptide Bacteriocin Plantaricin EF, *Master thesis, IMBV, Univ. Oslo*.

107. Kazazic, M., Nissen-Meyer, J., and Fimland, G. (2002) Mutational Analysis of the Role of Charged Residues in Target-Cell Binding, Potency and Specificity of the Pediocin-like Bacteriocin Sakacin P, *Microbiology 148*, 2019-2027.

108. Fimland, G., Eijsink, V. G. H., and Nissen-Meyer, J. (2002) Mutational Analysis of the Role of Tryptophan Residues in an Antimicrobial Peptide, *Biochemistry 41*, 9508-9515.

109. Fimland, G., Johnsen, L., Axelsson, L., Brurberg, M. B., Nes, I. F., Eijsink, V. G. H., and Nissen-Meyer, J. (2000) A C-Terminal Disulfide Bridge in Pediocin-like Bacteriocins Renders Bacteriocin Activity less Temperature Dependent and is a Major Determinant of the Antimicrobial Spectrum, *J. Bacteriol. 182*, 2643-2648.

110. Oppegård, C. (2005) Analysis of the Two-Peptide Bacteriocins Lactococcin G and Enterocin 1071 by the Use of *in vitro* Site-Directed Mutagenesis, *Master thesis, IMBV, Univ. Oslo*.